experimental
ATHEROSCLEROSIS

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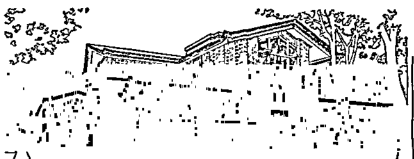
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To the Memory of
Deborah V. Dauber, M.D.

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L.N.K.

J.S.

Chicago, Illinois

Levine, Department of Metabolic and Endocrine Research; Dr. Otto Saphir, Department of Pathology; Dr. Herbert Silverstone, Department of Cancer Research.

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FOREWORD

"But medicine has long had all its means to hand, and has discovered both a principle and a method, through which the discoveries made over a long period are many and excellent, and through which full discovery will be made, if the enquirer be competent, conduct his researches with knowledge of the discoveries already made, and make them his starting point."

On Ancient Medicine,
A Hippocratic Tract, Author Unknown, Circa 450 B.C.
Cited by B. Farrington,
Greek Science, Its Meaning for Us (Thales to Aristotle), Penguin Books, Harmondsworth, Middlesex, England, 1944.

"Their obscurity [of the internal diseases], however, does not mean that they are our masters, but as far as is possible they have been mastered, a possibility limited only by the capacity of the sick to be examined and of researchers to conduct research. . . .

"When this information is not afforded, and nature herself will yield nothing of her own accord, medicine has found means of compulsion, whereby nature is constrained, without being harmed, to give up her secrets; when these are given up she makes clear to those who understand the art what course ought to be pursued."

The Art,
A Hippocratic Tract, Author Unknown, Circa 450 B.C.

I INTRODUCTION

ATHEROSCLEROSIS AND ARTERIOSCLEROSIS

THIS MONOGRAPH DEALS ONLY WITH ATHEROSCLEROSIS. A discussion of *other types of arteriosclerosis* is beyond its scope.

This subject delineation is not an arbitrary or artificial one. We emphasize the concept that the designation, *arteriosclerosis*, refers to several distinct pathologic entities. It is a generic term. It encompasses a number of different morbid processes, all of which produce thickening of the vessel wall. It includes alterations not necessarily associated with narrowing of the lumen, e.g., Mönckeberg's sclerosis.

When Lobstein first invented the term arteriosclerosis in 1833 (1), he used it precisely in this generic way. In his initial report on medial calcinosis (1903), Mönckeberg stressed that it was a pathologic entity separate and distinct from intimal arteriosclerosis (atherosclerosis) (2). In 1906 Klotz re-posed this problem of definition and classification: "What is arteriosclerosis? (a) is it an entity; or (b) are several distinct morbid conditions included under this one heading . . . ?" (3). He gave a clearcut answer to his question: "Let us preserve the broader meaning, and regard all scleroses or hardenings of the arteries as included under this general term, recognizing, if need be, distinct varieties" (3).

This view is still maintained by contemporary clinicians, pathologists and experimentalists (4–11). It has been reenforced by the data of Sappington and Cook (12). These workers studied various types of arteriosclerosis (diffuse in-

"Knowledge and Human power are synonymous, since the ignorance of the cause frustrates the effect. . . .

"Now the true and lawful goal of the sciences is none other than this: that human life be endowed with new discoveries and powers."

Novum Organum,
Francis Bacon, 1620.

" 'Tis *Works*, not *Words: Things*, not *Thinking: Pyrotechnic*, not *Philology: Operation*, not merely *Speculation*, must justify us Physicians. Forebear then hereafter to be so wrongfully Satyrical against our Noble Experimentators, who questionless are entred into the right way of detecting the Truth of things."

George Thomson,
London, 1671.
Cited by B J Stern,
Society and Medical Progress,
Princeton University Press, Princeton, New Jersey, 1941.

At the time Mönckeberg, Marchand and Klotz wrote, atherosclerosis had not yet been produced experimentally in any animal species. Experimental attempts with adrenalin, digitalis, barium chloride, pathogenic bacteria and other agents had produced arterial lesions, but not atherosclerosis. The changes in the animals' vessels resembled rather the Mönckeberg type of arteriosclerosis (medial calcinosis, senile arteriosclerosis) of man (3, 4, 14, 15).

In 1908, Ignatowski (16, 17) and Saltykow (18) first successfully induced true atherosclerosis experimentally by feeding rabbits diets of meat, milk or eggs. In these initial studies it was not clear which dietary factor was responsible for the lesions. Protein was particularly implicated. Stukkeu (19–21) and Wesselkin (22) soon demonstrated that only cholesterol-containing foods were atherogenic. Anitschkow (23–26) and Wacker and Hueck (27) induced typical lesions by giving rabbits pure cholesterol in oil, thereby proving conclusively that the sterol was in fact the atherogenic stimulus. Early reviews of this pioneering work were assembled by Dewey (28), Bailey (29), Chuma (30) and Schönheimer (31).

Experimental atherosclerosis as a field of research endeavor dates from these discoveries.

SIGNIFICANCE OF ATHEROSCLEROSIS

The importance of this research is emphasized by statistics on clinical morbidity and mortality resulting from atherosclerosis. Such data are not readily culled. Thus atherosclerosis is not included as an entity in the *International Lists of Diseases and Causes of Death* (32). Statistics on death rates due to "Arteriosclerosis" are exclusive of coronary and renal sclerosis (33). Coronary atherosclerosis is included under the heading, *Diseases of the Coronary*

timal thickening, Mönckeberg's medial sclerosis, focal intimal atherosclerosis) in the radial, coronary, splenic, renal and cerebral arteries at different ages. Neither Mönckeberg's medial sclerosis nor diffuse intimal thickening was related to the presence or absence of atherosclerosis in the various arterial beds. Boyd recently emphasized such facts and indicated the errors arising from their neglect: "One reason why a perusal of the discussions in the literature is so confusing is that a number of different lesions are included under the one name of arteriosclerosis, so that now one, now another condition is being alluded to. Many authors use the term as synonymous with atheroma. This is quite justifiable as long as the reader is familiar with this usage. It is not really a disease but is rather what Clifford Allbutt calls an omnibus name including several main divisions, the anatomical result of several morbid processes . . . Accordingly we shall not consider the general condition known as hardening of the arteries, but the subject will be divided into three sections: atherosclerosis, Mönckeberg's sclerosis, and diffuse arteriolar sclerosis" (5).

Hueper has recently presented a more detailed breakdown of the various pathologic types of arterial "hardening" (4). Obviously final decision concerning the merit of one or another of these classifications must await more precise elucidation of the pathogenesis and etiology of each process. Nevertheless, sufficient knowledge is currently extant to validate our identifying several distinct entities among the arterioscleroses. And for the sake of clarity the use of the plural term, the *arterioscleroses*, may not be without value.

In 1904 Marchand coined the term, *atherosclerosis*, to designate the type of intimal arteriosclerosis which is the subject of this monograph (13). The prefix athero (Greek *athere*, meaning mush) was selected to designate the amorphous lipid accumulation in the intima which is the hallmark of the developed lesion.

TABLE II

Crude Appraisal of Deaths Due to Atherosclerosis, Included in the Official International Lists of Causes of Death Under Various Headings

Cause of Death Official Headings	Approximate Number of Deaths Annually 1946–1948*	Our Estimation of the Number of Deaths Due to Atherosclerosis Annually
Diseases of Coronary Arteries and Angina Pectoris	150,000	120,000
Intracranial Lesions of Vascular Origin	130,000	75,000
Arteriosclerotic Kidney	55,000	5,000
Arteriosclerosis (except coronary or renal sclerosis)	25,000	5,000
Diabetes Mellitus	35,000	15,000

*Taken from Table I (33).

TABLE I

Deaths and Crude Death Rates from Several Causes—United States, 1946–1948 (33)

Cause of Death	Number of Deaths			Death Rates*		
	1946	1947	1948	1946	1947	1948
All Causes	1,444,337	1,445,370	1,395,617	988.5	1007.9	997.6
Diseases of Coronary Arteries and Angina Pectoris	160,520	154,131	137,153	109.9	107.5	98.0
Intracranial Lesions of Vascular Origin	131,036	131,039	125,646	89.7	91.4	89.8
Arteriosclerotic Kidney	55,407	56,691	56,095	37.9	39.5	40.1
Arteriosclerosis (except coronary or renal sclerosis)	25,629	25,317	24,918	17.5	17.7	17.8
Diabetes Mellitus	38,638	37,515	34,731	26.4	26.2	24.8

*Per 100,000 of population.

Arteries and Angina Pectoris;* cerebral atherosclerosis, under *Intracranial Lesions of Vascular Origin*; renal atherosclerosis, under *Chronic Nephritis* (a) *Arteriosclerotic Kidney* (33). The listing, *Diabetes Mellitus*, almost certainly includes many diabetics who succumb to atherosclerotic complications of this disease. The number of deaths and the death rates due to the foregoing causes during the years 1946–48 are listed in Table I (33).

Diseases of the coronary arteries and angina pectoris alone consistently account for over 10% of deaths annually. *At least 85% of these victims of coronary disease succumb to coronary atherosclerosis* (6–8, 34–37). Hence coronary atherosclerosis alone annually takes a toll nearly as large as all malignant tumors (33). Further, a rough appraisal is possible of the number of deaths actually due to atherosclerosis among the other listings in Table I. Katz and Dauber made such an estimate from the 1940 mortality statistics (38). We have repeated this analysis for the years 1946–48, making even more conservative estimates of the incidence of atherosclerosis (Table II). These figures indicate that atherosclerosis is the leading cause of death, claiming at least 200,000 victims annually in the United States of America, or a minimum of one-seventh of those dying from all causes. Almost certainly the true figure is higher (38–41), as is indicated by a recent National Heart Institute estimate (Table III) (575).

"AGING—INEVITABILITY" DOGMA

Obviously advances in the prevention and treatment of atherosclerosis are of great import for mankind. Their

*This listing of angina pectoris as a cause of death persists, despite the general recognition in clinical medicine that angina pectoris is a symptom, not a disease. It is a symptom of coronary artery insufficiency, which may result from a variety of morbid processes, the principal one being atherosclerosis (607).

TABLE III

Distribution of Causes of *Death* U.S. 1950 and 1951—National Heart *Institute* Circular (575)

	Number		Percent	
	1950	1951	1950	1951
All Causes	1,450,000	1,481,000	100.0	100.0
Cardiovascular Diseases	745,000	767,000	51.4	51.8
All other Causes	705,000	714,000	48.6	48.2
Total Cardiovascular	745,000	767,000	100.0	100.0
Arteriosclerosis	354,000	375,000	47.5	48.9
Arteriosclerotic H.D. incl. *Coronary* Dis.	323,000	338,000	43.4	44.1
General Arteriosclerosis	31,000	37,000	4.2	4.8
Hypertension	97,000	97,000	13.0	12.6
Hypertension with H.D.	84,000	83,000	11.3	10.8
Hypertension without mention of Heart	13,000	14,000	1.7	1.8
Mixed Hypertension and Arteriosclerosis	226,000	227,000	30.3	29.6
Vascular lesions affecting C.N.S.	156,000	158,000	20.9	20.6
Myocardial Degeneration	70,000	69,000	9.4	9.0
Rheumatic	22,000	23,000	3.0	3.0
Rheumatic Fever	2,000	1,000	.3	.1
Chronic Rheumatic H.D.	20,000	22,000	2.7	2.9
Other Cardiovascular	46,000	45,000	6.2	5.9

CVD Causes Over *Half of All Deaths*

Arteriosclerosis and Hypertension Cause Over 90% of all *Deaths from* CVD

product of senescence. The fact that young people may fall victim to its lesions demonstrates that aging is not essential for atherogenesis. Senescence and atherogenesis are two distinct and not necessarily interrelated processes. Data from the experimental laboratory re-enforce these conclusions.

The persistence of this dogma stems partially from a failure clearly to recognize the significance of the fact that arteriosclerosis is a generic term, embracing a number of distinct entities, among them atherosclerosis. This fact is today acknowledged by some proponents of the senescence "theory." Thus Moschowitz in a recent paper entitled *Hyperplastic Arteriosclerosis Versus Atherosclerosis*, labeled a "misconception" "the alleged identity of arteriosclerosis with atherosclerosis" (11). He then went on to advance a modified senescence theory, namely that "hyperplastic arteriosclerosis" (". . . characterized by hyperplasia of the intima, the internal elastic layer, and hypertrophy of the media . . .") is the "inevitable destiny of all animals that possess a vascular system such as the human one and who live long enough" (11).

In making this qualification, Moschowitz unfortunately did not fully clarify his concept of the relationship of atherosclerosis to this senescent process, hyperplastic arteriosclerosis. Certainly all investigators agree that the arteries, along with all living tissue, are subject to the biologic processes of senescence. Possibly "hyperplastic arteriosclerosis" is the morphologic end-product of these aging processes, as Moschowitz maintains. However, among the arterioscleroses, it is not hyperplastic arteriosclerosis that is the great human killer. *It is atherosclerosis.* It is the lipid-laden intimal plaques of atherosclerosis which impinge upon the lumina of the larger arteries, impairing the blood supply to heart, brain and kidney and bringing in their wake chronic illness and death. In focussing attention on hyperplastic arteriosclerosis, Moschowitz glosses over this prime fact. Further,

achievement would appear to be a high priority project for medical research. This, however, has by no means been the case. Until very recently, experimental atherosclerosis was a "stepchild" of medical research. It commanded very limited resources of personnel, equipment, plant and money. This situation, only partially improved upon at present, is a consequence of several circumstances. Among them, one of the most important is a fundamentally erroneous concept developed in the medical profession itself—the senescence "theory" of the genesis of arteriosclerosis. The thesis of this "theory" is a simple one: arteriosclerosis is the inevitable result of physiological aging processes. As such, it occurs unavoidably with advancing years As Askanazy put it, fully developed arteriosclerosis is the senile face of the arteries (42).

The stagnating influence this "theory" exercised on medical research is not difficult to imagine. In the words of Aschoff, "If arteriosclerosis were merely a phenomenon of aging, neither remedies nor prophylactics would be of any avail, for no one can escape age and death" (43). This atmosphere of helplessness and hopelessness engendered by the senescence "theory" was for many years a serious brake on investigation. Why attempt to prevent or cure the "inevitable"! Even today this sense of defeatism and futility, borne of the senescence "theory," remains to handicap and retard us This dogma, like others, dies hard!

It persists today in certain quarters even in the face of indisputable postmortem evidence demonstrating that clinically significant, marked atherosclerosis (among the arterioscleroses) undoubtedly occurs in some very young people and undoubtedly is minimal in some very old people (4, 35, 37, 38, 40, 41, 44–51, 227, 576). This is true for certain other types of arteriosclerosis as well (4, 12, 36). The fact that aged persons may be almost free of its lesions at autopsy demonstrates that atherosclerosis is not an inevitable by-

product of senescence. The fact that young people may fall victim to its lesions demonstrates that aging is not essential for atherogenesis. Senescence and atherogenesis are two distinct and not necessarily interrelated processes. Data from the experimental laboratory re-enforce these conclusions.

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by stressing his senescence theory of the pathogenesis of hyperplastic arteriosclerosis, he neglects the probability that atherosclerosis (admittedly a different entity from hyperplastic arteriosclerosis) is *not* a senescent process and hence is not inevitable.

Moschowitz's evaluation of the pathogenesis of atherosclerosis and of the significance of experimental cholesterol-induced atherosclerosis is equivocal: "There is no question that both grossly and histologically these experiments reproduce some of the characters of human arteriosclerosis, especially in respect to distribution of the lipid deposits. However, there are many points of difference*. . . In summary, no proof has been forthcoming that cholesterol 'arteriosclerosis' is identical with human arteriosclerosis . . . Nevertheless Anitschkow deserves credit for having done much to clarify the mechanism whereby *lipid deposition* or atheroma arises in human arteriosclerosis . . . At best Anitschkow has produced atherosclerosis but not arteriosclerosis" (11).

Undoubtedly Anitschkow produced atherosclerosis. Undoubtedly experimental cholesterol-induced atherosclerosis clarifies the mechanism of lipid deposition and atheroma formation in man. How does it clarify it? By demonstrating *that atherogenesis is consequent upon an alteration in lipid metabolism*. It is not a mere byproduct of senescence per se. As we shall demonstrate in this monograph, the evidence currently available indicates that without an altered lipid metabolism, little or no atherosclerosis will develop, regardless of any other alterations in the arterial wall, including senescent changes.† But it is precisely atherosclerosis (not

*Subsequently we shall consider the criticisms of experimental work and of the conclusions drawn from it concerning the pathogenesis of human atherosclerosis (cf. pages 134, 258, 280, 290) (L.N.K. and J.S.).

†This concept does not ignore the fact that in the older age groups atherosclerosis is present with increasing frequency and severity (cf. pages 65–67, 71, 79).

the other arterioscleroses) that is responsible for tremendous morbidity and mortality. If these atherosclerotic lesions are the result—or even only in part the result—of altered lipid metabolism, then they are not inevitable. The whole foundation of the senescence "theory" is rendered untenable. A hopeless situation is changed to one full of promise. A productive concept is substituted for one absolutely sterile. The possibility, nay *inevitability* presents itself that preventing or reversing the altered lipid metabolism will eliminate atherosclerosis. Hyperplastic arteriosclerosis may perhaps persist,* but tremendous morbidity and mortality certainly will not—for these are sequelae of atherosclerosis (586).

The key task of research in this field then is precisely to [illegible] lations between altered [illegible] Thereby we will forge [illegible] inating atherosclerosis. We have devoted considerable attention to the senescence "theory," *precisely because this achievement is impossible until this dogma is finally laid low.*

METHOD AND PURPOSE OF RESEARCH

Over and above its misinterpretations of factual data, the senescence "theory" is fundamentally unsound theoretically and methodologically. It is grounded on a conceptual fallacy which contemporary medical research must be on guard

*This too must be classified for the present as a moot question, particularly [illegible]

[illegible] may eventually be rendered amenable to rational prophylaxis and/or therapy.

against, particularly in dealing with the so-called degenerative diseases. This fallacy is the view that the task of science is merely to observe nature and record her workings, rather than to intervene actively in nature's processes and intelligently mold them according to man's designs.

Medical research finds itself not long out of the era of therapeutic nihilism Its thinking, particularly on the "degenerative" diseases, is still influenced by the negativistic doctrine that they are all "inevitable natural processes of aging." This viewpoint, strengthened in part by the slowness of research progress, is the contrary of the whole Baconian spirit of modern science. It is analogous to the ancient and medieval belief about epilepsy, brilliantly castigated by an unknown Hippocratic physician in the 5th century B.C., 2500 years ago: "It seems to me that the disease is no more divine than any other It has a natural cause, just as other diseases have. Men think it divine merely because they do not understand it But if they called everything divine which they do not understand, why, there would be no end of divine things" (52).

If one were to substitute the word, *inevitable* for *divine* in this quotation, it would be fully applicable to the senescence "theory." Undoubtedly *at the present stage of our knowledge* the arterioscleroses, including atherosclerosis, are "inevitable" processes of aging, at least in the United States, for they are today inevitably found with increasing frequency and severity in the older age groups. But this contemporary "inevitability" is one of scientific ignorance, and must be regarded as temporary. Just as the medical "inevitabilities" of 1900 have been negated, so will those of 1952. At that time, at that stage of medical progress, it was "inevitable" that the acute infectious diseases should be among the first causes of death, taking a toll of tens of thousands annually. Until Banting and Best discovered insulin, it was "inevitable" that thousands should die of the acute meta-

bolic derangements of diabetes mellitus. Today, in the insulin era, it is "inevitable" that thousands of chronic diabetics, maintained for years on insulin, die due to the atherosclerotic "complications" of this disease. Tomorrow this "inevitability" too will be cancelled out by the advances of medical science. Similarly, until 1949 it was "inevitable" that millions be debilitated, crippled and bedridden by the "degenerative" disease rheumatoid arthritis—until medical research developed cortisone and ACTH and seemingly negated another "inevitability."

Does not medical science in fact take this same positive historical approach even to the problem of true senescence itself, and its "inevitability"!

Man differs from other animals precisely in this ability consciously to transform nature according to his will. And this ability is itself limited at any given moment only by the level of man's scientific grasp of nature's processes. For it is this scientific understanding that enables him wittingly to manipulate nature according to his own designs.

Thus have human science, ingenuity and technique transformed the face of the earth. Nor has this process been confined to the inanimate world. In the course of his scientific activity, man has transformed himself as well—through technology in general and medical science in particular.* The whole written history of medical science, and particularly the epochal advance of the last 100 years, is essentially a process of man's altering his own "natural" biology, of transcending "inevitables."

In the "halcyon" days of ancient Rome man's life span was 20–30 years. In the decade 1879–1889 it had lengthened to 34 years. By 1911 it was 46.6 years. In 1944 it was 64 years! Scientists calmly project a life span of 100, and possibly even 140 years for the not-far-distant future!

*This transformation has of course had its negative unwitting aspects. Witness the so called "diseases of civilization."

against, particularly in dealing with the so-called degenerative diseases. This fallacy is the view that the task of science is merely to observe nature and record her workings, rather than to intervene actively in nature's processes and intelligently mold them according to man's designs.

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II HUMAN CLINICO-PATHOLOGY: BACKGROUND AND FOUNDATION OF EXPERIMENTAL ATHEROSCLEROSIS

In their current status as research problems, both experimental and clinical atherosclerosis are indissolubly linked with the problem of cholesterol, lipid and lipoprotein metabolism.* Both the considerable experimental work done during the last four decades and the most recent advances of clinical research emphasize the validity of this viewpoint. Evidence from investigations on man have a four-fold source: (1) histo-pathologic; (2) biochemico-pathologic; (3) ethno-pathologic; (4) clinico-pathologic. These findings constitute the firm background and foundation for experimental atherosclerosis. Hence they will be reviewed prior to the discussion of experimental atherosclerosis per se.

HISTO-PATHOLOGIC EVIDENCE

The pathologists of the second half of the nineteenth century recognized that extensive lipid deposition was a characteristic feature of atherosclerosis (70). In writings published almost 100 years ago (1856), Virchow, the father of cellular pathology, already used the term *atherosis* to describe this process (71). Even before this, Vogel identified cholesterol in atherosclerotic plaques (72) In 1857,

In 1850, only 8.9% of the American population was over 50 years of age; in 1930, 17.2%. In 1900, there were only 8,500,000 Americans in the age group 50–74; in 1940, 24,000,000; in 1980 it is estimated that there will be 42,000,000. Incidentally, it is precisely these self-transformations that have made atherosclerosis man's greatest medical problem, since—as already indicated—atherosclerosis is more frequent and severe in the older age groups.

The only real inevitability is the inevitability that in decades, centuries and millennia ahead man will continue this process of self-transformation. Science—guided by its Baconian creed—undertakes to make this a wilful, purposeful process, for the benefit of humanity. Inevitably, therefore, science must reject concepts like the senescence dogma, which are the very antithesis of this purposeful, humanistic activity. Inevitably science must come to grips with the major roadblocks to this process of fruitful self-transformation:—among the diseases, atherosclerosis, cancer, the true senescent processes themselves and others. Inevitably, the judicious application of the scientific method must—sooner or later—bring these under control. Inevitably, man must thereby further alter his own natural history and further transform himself biologically.

For us working in experimental atherosclerosis, confidence in this inevitable triumph of science is a most profound conviction and mainstay. To the achievement of this triumph over atherosclerosis particularly, our research and this monograph are dedicated.

TABLE IV

Cholesterol Content of Coronary Arteries in Cases Dying Coronary and Non-Coronary Deaths
—Morrison, L. M. and Johnson, K. D. (585)

Causes of Death	No. of Cases	Average Age	Cholesterol in Coronary Arteries mg %	Plasma Cholesterol mg %	Grade of Coronary Atherosclerosis
Acute coronary thrombosis	25	64	20.4	303	3.5
Non-coronary	25	62	5 1	186	1.5

Mettenheimer noted that the lipoidal mass was doubly refractive due to the presence of cholesterol esters (73). This fact was rediscovered by Aschoff in 1907 (74). Virchow advanced the concept that atherosis was a degenerative change (fatty imbibition and metamorphosis) secondary to inflammatory necrobiotic processes in the connective tissue cells and ground substance of the intima (75). The pathogenic significance of lipid deposition in the vascular wall has been a subject of sharp controversy ever since (4) (cf. also pages 76–86). The careful histo-pathologic studies of Leary have accumulated considerable data indicating that the intimal foam cell cushion (atheroma) is the initial lesion of atherosclerosis in man (7, 8).

BIOCHEMICO-PATHOLOGIC EVIDENCE

Early in the twentieth century, the findings of the pathologists were supplemented by biochemical studies. Windaus demonstrated that atheromatous aortas contain six to seven times as much free cholesterol, and 20–26 times as much cholesterol esters, as normal aortas (76). These observations were subsequently confirmed and extended by numerous investigators (38, 73, 77–86). Recently Morrison and Johnson further showed that the average cholesterol content of the coronary arteries of a group of patients dying from an acute coronary thrombosis was four times as great as the average cholesterol content of the coronary arteries in a comparable group of control patients (Table IV) (585). This finding constitutes further objective evidence in support of the concept that most cases of coronary disease are on an atherosclerotic basis. It also confirms the judgment that a group of people with clinical coronary disease have more atherosclerosis than a clinically normal group of similar age, sex and background (cf. the discussion of this problem in reference 567; see also reference 607 for definitive data).

rosclerosis was seen in males or females. Only seven among 150 cases exhibited any aorta sclerosis, and in no case was it severe. Coronary atherosclerosis was seen in only five older persons, and only one had calcification. The brains and hearts were devoid of atherosclerotic sequelae. Similar autopsy findings on Okinawans have also been reported by Benjamin (91).

These Okinawans were well-nourished and well-developed. They had subsisted on a good diet prior to the military action on their island. Their fare was predominantly vegetarian, being high in carbohydrate (sweet potatoes, rice, vegetables) and low in fat. They consumed little meat and milk; vegetables (soybeans) furnished most of the protein in their diet (50).

A number of reports are available concerning atherosclerosis among the Chinese. Autopsy data led Oppenheim to conclude that it is rare (51). Rosenthal's review confirmed this conclusion (80). More recently Snapper has further substantiated it (92). In general, all investigators described a diet among the Chinese that was low in protein, animal fat and calories.

A recent report by Cullumbine on cardiovascular disease in Ceylon is consistent with the foregoing observations (571). Cullumbine found an extremely low death rate from cardiovascular disease in Ceylon (20–30 per 1,000 deaths; cf. Tables I, II and III). "There is a racial difference in incidence of cardiovascular disease. The Dutch burghers are largely responsible for the incidence of deaths from cardiovascular disease. The more indigenous races, the Sinhalese and the Malays and Tamils don't die as readily from cardiovascular thrombosis as do the burghers. Economic status is very important. We find the higher the economic status of the people, the greater the death rate from cardiovascular disease" (571). The low death rate from cardiovascular disease in Ceylon is accounted for only in small part by the low

ETHNO-PATHOLOGIC EVIDENCE

Over the years considerable data have been accumulated indicating that differences exist among peoples in incidence and severity of atherosclerosis. These differences tend to correlate with culturally-conditioned variations in nutrition and diet, rather than with racial, climatic or other factors. Thus Rosenthal's analysis of 28 reports up to 1934 generally showed: ". . . . In no race for which a high cholesterol intake (in the form of eggs, butter and milk) and fat intake are recorded is atherosclerosis absent . . . . Where a high protein diet is consumed, which naturally contains small quantities of cholesterol, but where the neutral fat intake is low, atherosclerosis is not prevalent" (80).*

A. Costa Ricans, Chinese, Japanese, American and African Negroes, Kirghiz Plainsmen, Eskimos, Ceylonese, Okinawans

Evidence accumulated since Rosenthal's review is in general confirmatory (4) For example, both Anitschkow (89) and Wilens (90) noted that the inhabitants of Costa Rica showed few or no atheromatous plaques in the aorta, coronary or other arteries. This colonial people is generally malnourished, subsisting on a diet low in animal protein and fat.

Steiner has recently reported on necropsy findings in Okinawans (50). He found these people were amazingly free of "degenerative" disease of the cardiovascular system. No hypertensive cardiovascular disease or malignant neph-

*The report of Rogers is an exception (87). He could find no definite evidence that atherosclerosis was less frequent in Calcutta than in London, [illegible] animal protein and fat. [illegible] and Rai observed 50% [illegible] (80, 88). The Indians [illegible] little butter, milk and [illegible] reflect caste and class [illegible] This possibility merits exploration. Plasma lipid findings in Indians are discussed in a subsequent section of this monograph (pages 33 and 40).

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life expectation. Cullumbine further reports that fat intake is small in Ceylon, as is animal protein and total caloric consumption. Vegetable protein intake is fairly high. Level of fat intake varies with economic status, as does the death rate due to cardiovascular disease (571). Finally, De Zoysa (587) reports a low incidence of atherosclerotic sequelae of diabetes mellitus, an "extremely rare" disease in Ceylon (571).

The apparent lack of susceptibility of these peoples to atherosclerosis is not attributable to racial factors. Thus significant atherosclerosis occurs not infrequently among Chinese in the United States, subsisting on diets similar to that of the American people as a whole (92). Similarly, Negroes in the United States are frequent victims of atherosclerosis, whereas Negroes inhabiting the Kenya Colony in Africa rarely exhibit this disease (94). Like data are available concerning Japanese and Nisei (4, 80, 93).

In studies on an Asiatic people at the opposite end of the dietary spectrum, Kuczynski recorded a high incidence of obesity, premature extensive atherosclerosis, contracted kidney, apoplexy and arcus senilis in the nomadic Kirghiz plainsmen (95). These people consumed unusually large amounts of milk and meat. Their kinsmen, who had been urbanized and subsisted on a more moderate and varied diet, did not exhibit such severe vascular disease

Considerable interest has also focussed upon findings in Eskimos. Almost 30 years ago, Krogh and Krogh studied the dietary habits of Eskimos in southern Greenland (96). These people, as well as Eskimos in other locales (97–101), subsist mainly on large quantities of raw meat and fish. Considerable controversy exists as to the amount of fat in such diets. Thomas stated that the Eskimos of North Labrador and Greenland eat little fat and blubber, since the lipid is used for light and fuel (97). Krogh and Krogh estimated that the raw meat eaten by South Greenland Eskimos contained 6–10% fat, and their daily diet in times of plenty

consisted of about 280 grams of protein, 135 grams of fat and 54 grams of carbohydrate (96). Heinbecker observed that the "lean meat" eaten by Baffin Island and Greenland Polar Eskimos contains considerable quantities (approximately 1/7th) of fat. These people consume four to eight pounds of such meat daily when it is available (98). On such diets Eskimos remain free of ketonuria; they apparently develop an unusual capacity to oxidize fat (98). Their basal metabolic rates are high by the usual standards. Recently Alexander described the diet of Aleuts as consisting chiefly of fish and seal meat, sea-lion meat, duck, geese, reindeer meat, blubber and very little vegetables, green foods, fruit or milk. It is a diet relatively high in protein, low in carbohydrate and moderate in fat, with a low total caloric intake ranging from 800 to 1400 calories per day (588).

In summary, it would appear that Eskimos ingest moderate amounts of cholesterol contained in animal muscle and viscera. In addition, they consume not inconsiderable quantities of neutral fat, although their reputed intake of huge quantities of "blubber" is erroneous. Finally, the foregoing facts are valid for periods when food is plentiful. Such times almost invariably alternate with periods of semistarvation.

Little data are available concerning the incidence and severity of atherosclerosis in Eskimos. Thomas stated that they exhibited no unusual prevalence of vascular or renal disease (97). Perusal of his paper reveals that he focussed attention on blood pressure levels and urinary findings as criteria of cardiovascular-renal disease. The actual incidence and severity of atherosclerosis in Eskimos cannot be estimated from Thomas' observations. Alexander found hypertension to be practically non-existent among Aleuts (588). His electrocardiographic and clinical studies of 296 male and female Aleuts of all ages, including 23 above age 60 (13 males and 10 females), revealed almost no cardiovascular-

renal disease. Garn and Gertler's report is similar (589). More complete data are not available from other sources. To our knowledge, no workers report autopsy findings in Eskimos. Apparently further data are essential before overall valid estimates can be made of the comparative incidence and severity of atherosclerosis among different tribes and groups of Eskimos. At present, therefore, it would not appear justified to regard findings in Eskimos as exceptional to those in other peoples, wherein a general relationship is demonstrable between level of caloric and lipid ingestion, and frequency and severity of atherosclerosis.

B. World Wars I and II

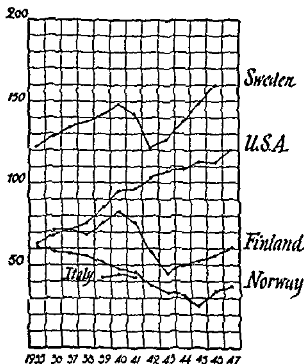
Several studies on people living in Western countries furnish further leads on the possible relationship of diet to atherosclerosis. Among these are reports of the effects of wartime dietary austerity on incidence and degree of atherosclerosis. Aschoff observed a lower incidence of atherosclerosis in Central Europe after World War I. He attributed this to the low level of dietary fat ingestion (102). Beitzke noted a like finding in autopsy material during these years of fat-poor nourishment in Germany (103). Similar data are becoming available on the effects of World War II. Thus Malmros has recently analyzed pre-war and wartime death rates due to arteriosclerosis in several European countries (104). His salient findings are summarized in Figures 1 and 2, taken from his paper These data indicate clearly that peoples suffering from fascist-imposed dietary restriction exhibited a definitive decrease in deaths due to arteriosclerosis. Recent reports from Norway, Finland and Russia further confirm and extend these observations (Figs 3, 4 and 5) (537, 537a, 570, 590, 591).

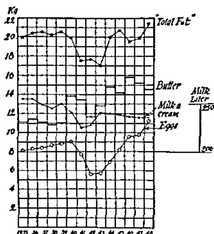
Consistent with these observations implicating dietary lipid in the pathogenesis of atherosclerosis is Dock's impression that "the incidence of coronary disease in men under

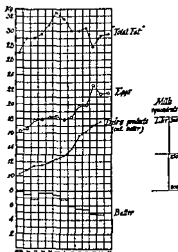
35 years of age appeared to be relatively high in the Ar can military population, in training in the United State compared with the British in Britain, whose intake of butter, ice cream, milk and cream was much lower" (This impression implicating exogenous lipid as a dec factor in atherogenesis in young men remains to be ver (cf. footnote, p. 31).

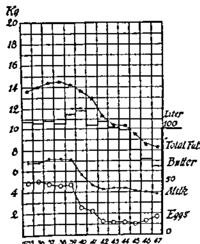
C. Under- and Overnutrition

In studies on a general European population, Grot al. found that the degree of atherosclerosis paralleled









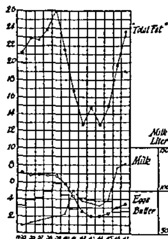


Fig. 2. Consumption of eggs, dairy products and total fat in Sweden, U.S.A., Finland and Norway, (1935–1947)—Malmros, H. (104).

quality of nutrition (105). Atherosclerosis was most severe in people who ate excessively, particularly of foods rich in cholesterol. The accumulated findings of American life insurance companies lend further weight to this conclusion. The data of Dublin are particularly revealing (40). Table V has been abstracted from his paper. Dublin concludes: "Considering the overweights first, because their excess mortality is very large for all causes, we find that at all ages combined, they show exceptionally high death rates from organic heart disease and angina pectoris, arterial diseases, diabetes, nephritis and cerebral hemorrhage. . . ." (40). * The *extreme overweights show even higher death rates due* to these diseases. Further, all age groups exhibited these trends. However, they were significantly more marked among older overweights. Several other studies report essentially similar findings (592–594).

Wilens' recent analyses of autopsy data also demonstrate

*In the Introduction, we reviewed the reasons validating the concept that a high percentage of deaths listed under the above official headings are actually attributable to atherosclerosis.

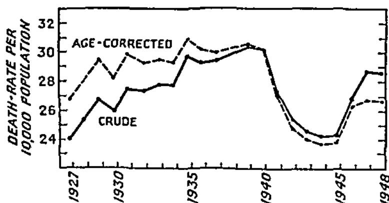
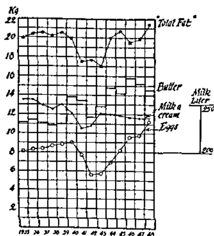
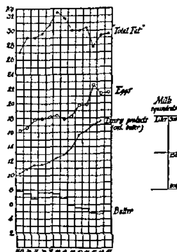


Figure 3 Mortality from circulatory diseases in Norway in 1927–48. Standard population = population of Norway in 1940—Strøm, A. and Jensen, R. A. (537a).

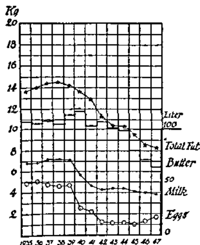
Sweden



U. S. A.



Finland



Norway

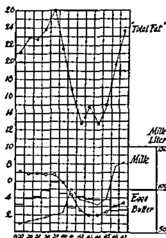


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the influence of general nutritional state on atherogenesis (90, 106). In one investigation, he found that less atherosclerosis is present postmortem in people dying after a prolonged debilitating illness than in people succumbing acutely (106). The data of his second study on obesity and atherosclerosis indicate that advanced atherosclerosis is about twice as common in obese as in poorly nourished persons. Almost twice as many of the poorly nourished group

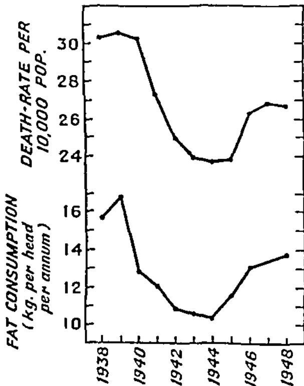


Figure 5. Mortality from circulatory diseases, corrected for age; consumption of fat in form of butter, milk, cheese, and eggs—Strøm, A. and Jensen, R. A. (537a).

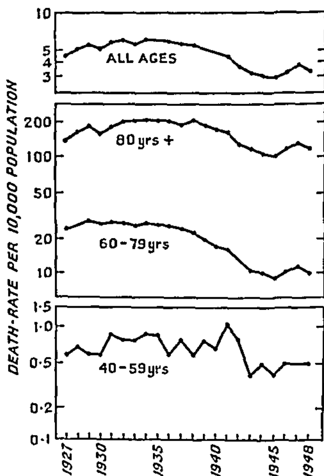


Figure 4. Mortality from arteriosclerosis in Norway in 1927–48, by age-groups—Strøm, A. and Jensen, R. A. (537a)

had little or no atherosclerosis at autopsy. In both these respects, the group with average nutrition was intermediate between the poorly nourished and obese groups. This relationship between state of nutrition and atherosclerosis was independent of age, sex, hypertension, heart weight and diabetes (90). Clearly, data from several sources indicate a relationship between obesity and atherogenesis. However, contradictory findings have been reported by several investigators. Thus Faber and Lund analyzed the influence of obesity on aorta atherogenesis, utilizing aorta dry weight, calcium and cholesterol content as objective indexes of sclerosis (595). With these criteria, they noted no correlation between obesity and atherosclerosis, when account was taken of the presence of hypertension. Similar negative results were also obtained by Rosenthal (80), by Levy, *et al.* (596), and by Garn, *et al.* (597). The last group of workers compared 97 males under the age of 40 with a myocardial infarction and 146 comparable normal controls. The coronary group was no more overweight than the control group (597). Finally, contrary to French and Dock's initial report (44), the more complete study of Yater, *et al*, of coronary atherosclerosis in American soldiers of World War II led to this conclusion: "Therefore, it appears that obesity cannot be said to be an etiological factor of any importance" (35).*

Patently, ethno-pathologic data relating diet, nutritional state and atherogenesis are circumstantial, incomplete and in part contradictory. Nevertheless, at least a tentative con-

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*A number of these studies yielding negative results were done on young persons with coronary atherosclerosis (35, 597) As Faber and Lund point

TABLE V

Standardized Death Rates per 100,000 for Specified Causes of Death—All Ages Combined—by Weight Classes—Dublin, L. I. (40)

Causes of Death	Death Rates per 100,000		
	Underweight Class	Normal weight Class	Overweight Class
All Causes	848	844	1111
Organic Diseases of Heart	65	80	121
Angina Pectoris	14	16	35
Diseases of the Arteries	17	23	38
Nephritis, acute and chronic	63	82	141
Cerebral Hemorrhage and Apoplexy	49	70	110
Diabetes Mellitus	9	14	36

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Patently, ethno-pathologic data relating diet, nutritional state and atherogenesis are circumstantial, incomplete and in part contradictory. Nevertheless, at least a tentative con-

*A number of [illegible]

. . . d on the no-

osclerosis is generally more frequent and severe in well-nourished, particularly over-nourished, people subsisting on diets rich in animal fat. Certainly, as Weiss and Minot warned in 1933, "Caution is necessary at this point lest we fall into the so often dangerously applied post hoc . . . these evi-

compel a revision of their opinion, "There is no proof that overnutrition leads to arteriosclerosis in man" (112).

D. Diet and Cholesterolemia

How does nutritional state influence atherogenesis? The cholesterol concept hypothesizes that diet induces alterations in lipid metabolism which plays a key role in the development of arterial lesions.* Unfortunately, few of the foregoing ethno-pathologic reports contain data on lipid metabolism. For example, data on plasma cholesterol levels in the Chinese and Okinawans, or in underweight and overweight persons, were not published, although Snapper stated that plasma cholesterol levels are low in Chinese (92). Adolph described the Chinese diet as being essentially vegetarian, with about 90% of the caloric intake in the form of cereals (660, 661). The amount of meat or animal food is generally very small; only about 5% of the protein intake is animal protein. Practically no milk or milk products are . . . adult Japanese subsist- . . . or many years, revealed a plasma cholesterol level of 140 mg.%, and low S_f 12–20 levels (657) (cf. pages 42 and 92, et seq.).

Only limited, contradictory data are available on plasma

*As indicated in the preceding footnote, the cholesterol concept does not hypothesize that diet (exogenous cholesterol) is the only factor in atherogenesis.

cholesterol concentrations among the peoples of India. Ghose (113), and Boyd and Ray (114) report that the plasma cholesterol levels of Indians are significantly below values accepted as normal standards for Europeans and Americans. In contrast, Bose and De conclude that "the cholesterol content of the blood (plasma) in healthy normal Indian subjects does not differ materially from the standard European and American figures" (115). Here again, overlooking class- and caste-conditioned differences in dietary habit may have contributed to contradictory results.

Plasma lipid studies on Eskimos have also yielded inconsistent data. Corcoran and Rabinowitch studied two groups of Canadian Eskimos, one on a mixed diet, the second subsisting exclusively on meat (99). The latter tended to have higher plasma lipid levels. However, both groups exhibited plasma lipid and cholesterol concentrations lower than the normal values recorded in Americans with the analytical methods used (99, 116, 117). Corcoran and Rabinowitch suggested that this phenomenon may be related to the high B.M.R. of Eskimos (99, 118). Sinclair, *et al.*, published plasma lipid findings in Eastern Canadian Arctic Eskimos that parallel closely values regarded as normal in Americans (54, 100, 117). In contrast, Wilber and Levine found the plasma lipid levels of Eskimos residing in Point Barrow, Alaska to be moderately elevated, compared with accepted normal values for the methods employed (101, 117). Alexander reported mean plasma cholesterol levels of 176 and 197 mg.% in two groups of Aleuts (588); Garn and Gertler obtained a value of 204 mg.% in Aleuts over 30 years of age (589).

What other data are available on the effect of nutritional state in general, and cholesterol ingestion in particular, on cholesterol levels and lipid metabolism in man? Fortunately,

enough data are extant to permit at least a tentative answer to this question. In an extensive review, Weinhouse concluded that the plasma cholesterol concentration is not influenced by diet (55). Peters and Van Slyke also stated that it is not readily affected by variations in cholesterol intake (54). *Within definite limits,* these conclusions would appear to be valid. Thus recent studies of Steiner (9), Keys (119) and Gertler, *et al.* (111), are all confirmatory. Gertler's studies of men under 40 with and without coronary atherosclerosis led to the conclusion that in neither group was there a relationship between the amount of cholesterol ingested and the level of cholesterolemia (111).* Steiner and Keys did acute and subacute studies in man. In about 60 acute experiments, Keys found that ingestion of 10 grams of cholesterol with ample fat resulted in only a slight temporary hypercholesterolemia. Steiner observed no alimentary hypercholesterolemia following a lipid-laden breakfast in which 20 grams of cholesterol were included.† In further experiments Steiner placed patients alternately on diets containing 50 or 300 grams of fat for six-week periods. In five of nine patients no increase in total serum cholesterol occurred on the high fat diet; in four subjects slight hypercholesterolemia was noted. Subsequently three patients were given the high fat diet plus 10 grams of cholesterol in 200 cc. of milk Serum cholesterol levels were not significantly affected. Similarly, persons with coronary arteriosclerosis showed no significant alterations in serum cholesterol con-

*These workers found that a group of men under 40 with a history of myocardial infarction tended to exhibit moderately elevated plasma lipid and cholesterol levels, compared to a normal control group. This difference was present although the two groups ingested similar quantities of cholesterol weekly (3 88 ± 1.46 grams and 3.30 ± 1.41 grams) (107–111).

centration when placed on high (8 grams daily) and low cholesterol diets for six-week periods (9).

Steiner's high cholesterol diets contained more sterol than even extreme gourmands ordinarily ingest. Keys assessed the effects on plasma cholesterol of ordinary dietary variations in sterol ingestion (119).* He concluded that "the serum cholesterol level of 'normal' men as represented here is not significantly related to differences in the habitual cholesterol intake over a range of something like 250–800 mg. per day" (119). Consistent with this finding is Gofman's report that the pattern of plasma cholesterol-bearing lipoprotein molecules is not significantly influenced by dietary alterations within the usual range of the American diet (see below, pages 42 and 92, et seq.) (567).

Although this conclusion is apparently incontrovertible, it should not be taken to indicate the absolute unresponsiveness of plasma cholesterol to diet. On the contrary, further data suggest that other dietary alterations, somewhat different from the foregoing in type, degree or duration, raise plasma cholesterol levels of normal persons. Thus Sinclair, et al., found that exclusive consumption of pemmican, containing 75% beef fat, resulted in a rise of all plasma lipids (100). Similarly Tolstoi observed an increase in plasma lipids, including cholesterol, in man subsisting on an exclusive meat and fat diet for one year (124). Recently, Messinger, et al., showed that addition of egg yolk powder to the diet caused a significant rise in cholesterolemia in humans (338).† Egg yolk powder was more effective than pure

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*Keys estimated that the American diet varies in cholesterol content from a low of 200–300 mg. daily, to 700–800 mg, depending on foods ingested (119). Gubner and Ungerleider (57), citing Cook (121), gave the following figures for daily cholesterol intake: low fat diet—39–109 mg.; mixed diet—200–360 mg; fat-rich diet—up to 1400 mg. For data on the cholesterol content of foods, see references 80, 122, 123.

†The amounts of egg yolk powder needed to raise plasma cholesterol levels were well above those consumed in the ordinary diet (338).

TABLE VI

Effect of Rice Diet on Total Serum Cholesterol of 511 Patients with Hypertensive Vascular Disease
—Kempner, W. (125)

	Rice Diet		Average Period of Rice Diet (days)
	Before	During	
148 Patients with initial concentration below 220 mg %	186	171	120
363 Patients with initial concentration above 219 mg %	279	205	102

cholesterol in elevating plasma cholesterol levels. This suggested to Messinger, *et al.*, that egg yolk contains an as yet *unidentified factor which increases cholesterolemia.*

Keys' study also demonstrated that the plasma cholesterol level may be lowered by more extensive dietary changes (119). Hypertensive patients on a cholesterol-free, fat-free rice diet exhibited regular and rapid marked declines in serum cholesterol. Thus, in four subjects, the mean control total cholesterol value was 232.5 mg.%. After three weeks on the rice diet it had declined to 151.8 mg.%. Kempner's similar results are detailed in Table VI (125). Starke (126) and Watkin, *et al.*, (127) recently reported a like effect of the rice diet on cholesterolemia. Mellinkoff, *et al.*, report the same serum cholesterol response to a synthetic cholesterol- and fat-free diet composed of a protein hydrolysate and dextrimaltose (128). Keys' data are particularly significant in demonstrating that the fall in serum cholesterol is dependent on the absence of both cholesterol and neutral fat from the diet (119). When cholesterol-free vegetable fat was added to the rice diet, plasma cholesterol rose to control levels (cf. Gofman's similar observations, p. 42).* These observations need to be extended, since the relationship of neutral fat to cholesterol metabolism and atherogenesis is a key problem, inadequately understood at present (10, 53, 54, 57, 80, 86, 89). The combined tools of biochemistry, physiology and pathology need to be applied in both man and experimental animals for the unraveling of this important problem. Its significance is emphasized by the fact that—based on observations such as the foregoing—low fat-

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*[illegible] concentration (545).

TABLE VII

Changes in Plasma or Serum Lipids during Periods of Restricted Food Intake*—Keys, A. *et al.* (130)

Author	Reference Number	Subjects	Days of Restriction	Total Lipids	Lipid Phosphorus	Total Cholesterol
Feigl, J.	131	adults				o and +
Rosenthal, F. and Patrzak, F.	132	adults				—
Hetenyi, G.	133	adults	8	o	—	
Man, E. B. and Gildea, E. F.	129	adults				—
Bock, K. A	134	adults	14			
Man, E. B. and Gildea, E. F.	135	adults			o	o
Gounelle, H. et al.	136	adults		o		o
Block, M.	137	adults	28	+, —		—

Raynaud, M. and Laroche, C	138	adults		o	o	o
Hodges, R G. et al	139	children				—
Lambrechts, A et al	140	adults		o		o
Keys, A et al	130	adults	168			— and o
Poindexter, C A. and Bruger, M	141	obese	42-420			o
Hetenyi, G.	133	obese	8	—		
Block, M.	137	obese	35	+, —		
Coste, F et al.	143				—	
Nicaud, P. et al.	144				o	
Bose, J. P et al	145					o

* o = no change in concentration
\+ = a greater than normal concentration.
— = a lower than normal concentration.

low cholesterol diets are being used clinically to an increasing extent in the prophylaxis and therapy of human atherosclerosis (cf. also, references 556, 567, 658, 663, 708–710). As a recent review by Davidson indicated, further work is essential before the merit of such regimens can be finally ascertained (663).

Additional data are available indicating that plasma cholesterol levels are not unresponsive to nutritional factors. Man and Gildea found that malnutrition, in contrast to mere thinness, caused both plasma cholesterol and lipid phosphorus to fall (129). In their extensive study on *The Biology of Human Starvation*, Keys, *et al.* reviewed the extant data on the effects of chronic semistarvation on cholesterolemia (130). Their findings are summarized in Table VII, from their book. Data on people in Germany, France and Belgium during World War II, and on Indians during a Bengal famine are surveyed. The conclusion was reached, "The preponderance of evidence indicates that the concentration of cholesterol in the blood decreases during semistarvation. Such a change was conclusively established in the Minnesota Experiment" (130).

The findings in obese persons are more complex. Peters and Man reported that cholesterol did not parallel weight consistently (147). Further, obesity and hypercholesterolemia were not related. "The lipids of a group of obese adults lay in every respect within the normal range and were as often low as high" (147). Keys, *et al*, made a similar observation in a group of 200 middle-aged men (130). Except for the thinnest members of the group, who tended to have lower levels of plasma cholesterol, no correlation was noted between weight and cholesterolemia.* However, in a group of 180 young men, cholesterolemia did vary with

*Similarly, Gofman noted no correlation between obesity and patterns of plasma cholesterol-bearing lipoprotein molecules (see below, page 99, et seq.) (567).

relative obesity (130). Further, in obese persons repeated plasma cholesterol determinations revealed a tendency for the serum cholesterol to fluctuate considerably (147).* In contrast, other normal persons usually show little variation over long time periods in their serum cholesterol concentrations (54). Further, Keys, *et al.*, and Peters and Man noted that plasma cholesterol levels of obese persons fell following rigorous dietary restriction for reducing purposes (130, 147). These tendencies probably reflect the operation of endogenous regulatory mechanisms, as well as the influence of diet.

Obviously, the foregoing data on nutritional factors affecting cholesterolemia are far from complete. In such an ethno-pathologic survey, we would particularly like to analyze reports on plasma cholesterol levels and lipid metabolism in various peoples living under different socio-economic and cultural conditions. Only limited data of this kind are available. We would like to test further the possibility suggested by Gubner and Ungerleider, "that the so-called 'normal' cholesterol is in reality a high cholesterol, and that the cholesterol level of the average American population is of such an order as to predispose to the development of arteriosclerosis" (57) For the present, this review may be summarized, perhaps inadequately, in the words of Peters and Man: "Although serum cholesterol is frequently resistant to the immediate effects of food, it does not appear to be altogether unaffected by diet—or perhaps it would be more correct to say [by] the state of fat metabolism" (147).

Finally, it must be recognized that dietary and nutritional factors may influence cholesterol metabolism and atherogenesis without necessarily affecting the plasma total cholesterol concentration Recently we suggested· "It is not

*This tendency toward variability of the serum cholesterol level was found by Steiner, Morrison and others to be frequently associated with atherosclerosis (9, 148, 149).

only the *level* of cholesterol in the plasma that is important for atherogenesis, but also the *quantity* of exogenous cholesterol the body must *transport, turn over and metabolize*" (53). The latest studies of Gould and Taylor lend weight to this point. They showed that the level of exogenous cholesterol ingestion influences the rate of endogenous cholesterol synthesis from acetate in dog and rabbit (150, 151).

The findings of Gofman, *et al.*, are particularly significant in this regard (152, 153). By ultracentrifuge techniques, these workers identified a class of plasma cholesterol-bearing lipoprotein molecules (S_f 10–20 class) associated with atherosclerosis in man. The presence of these molecules was not directly correlated with the plasma total cholesterol concentration. Thus, *groups* of people with low plasma total cholesterol levels tend to exhibit low concentrations of S_f 10–20 lipoprotein molecules; however, for *individuals* the level of total cholesterol yields no information concerning S_f 10–20 molecule concentrations. These workers also showed that the levels of S_f 10–20 molecules may be influenced by diet. Gofman, *et al.*, placed persons exhibiting significant concentrations of these lipoprotein molecules on a diet partially restricting fat and cholesterol. The serum level of such molecules gradually fell over a period of weeks and months. This decrease occurred whether the total cholesterol concentration fell or not. With the addition of vegetable oil without cholesterol to the diet, concentration of S_f 10–20 molecules again rose, further indicating the metabolic interrelationships between neutral fat and cholesterol These effects were not functions of under- or overnutrition, since these patients were kept at constant weight during changes in dietary regimen (152, 153).

These findings would appear to give further support to the concept, derived from the bulk of ethno-pathologic evidence, that dietary and nutritional factors related to choles-

terol and lipid metabolism play an important role in atherogenesis.

CLINICO-PATHOLOGIC EVIDENCE

Alterations in cholesterol metabolism occur in a number of disease states, among them diabetes mellitus, hypothyroidism, the nephrotic syndrome, biliary obstruction, essential (familial) xanthomatosis Hypercholesterolemia is a finding common to all of these. So is premature, severe atherosclerosis. Moreover, evidence has recently accumulated indicating that patients with coronary atherosclerosis, without gross hypercholesterolemia, have subtle alterations in cholesterol metabolism (107–111, 152, 153).

These findings constitute the clinico-pathologic background and foundation for experimental atherosclerosis. Laboratory research has been most fruitful when related to these clinical facts. Undoubtedly this close interplay between *investigation in humans and animals will continue to* be a key basis for progress in this research field.

A. Diabetes Mellitus

Diabetes mellitus is statistically the most important clinical entity associated with disturbed lipid metabolism and a hypercholesteremic tendency.* Coincidentally, control of "degenerative" vascular disease is the key therapeutic problem in diabetes today (53, 155). The data on the occurrence of atherosclerosis in diabetes have been recently collated by Liebow and Hellerstein (156). They are summarized in Tables VIII and IX, taken from their paper. The mass of evidence demonstrating a markedly increased incidence and severity of atherosclerosis in diabetes is incontrovertible. Provided they have the disease long enough, diabetics will

*This fact is driven home by data indicating that the number of persons with diabetes in the United States is nearly 2,000,000 (154).

only the *level* of cholesterol in the plasma that is important for atherogenesis, but also the *quantity* of exogenous cholesterol the body must *transport, turn over and metabolize*" (53). The latest studies of Gould and Taylor lend weight to this point. They showed that the level of exogenous cholesterol ingestion influences the rate of endogenous cholesterol synthesis from acetate in dog and rabbit (150, 151).

The findings of Gofman, *et al.*, are particularly significant in this regard (152, 153). By ultracentrifuge techniques, these workers identified a class of plasma cholesterol-bearing lipoprotein molecules (S_f 10–20 class) associated with atherosclerosis in man The presence of these molecules was not directly correlated with the plasma total cholesterol concentration. Thus, *groups* of people with low plasma total cholesterol levels tend to exhibit low concentrations of S_f 10–20 lipoprotein molecules; however, for *individuals* the level of total cholesterol yields no information concerning S_f 10–20 molecule concentrations. These workers also showed that the levels of S_f 10–20 molecules may be influenced by diet. Gofman, *et al.*, placed persons exhibiting significant concentrations of these lipoprotein molecules on a diet partially restricting fat and cholesterol. The serum level of such molecules gradually fell over a period of weeks and months. This decrease occurred whether the total cholesterol concentration fell or not. With the addition of vegetable oil without cholesterol to the diet, concentration of S_f 10–20 molecules again rose, further indicating the metabolic interrelationships between neutral fat and cholesterol. These effects were not functions of under- or overnutrition, since these patients were kept at constant weight during changes in dietary regimen (152, 153).

These findings would appear to give further support to the concept, derived from the bulk of ethno-pathologic evidence, that dietary and nutritional factors related to choles-

TABLE IX

Coronary Thrombosis—Occurrence in Diabetics and Non-diabetics at Postmortem—Liebow, I. M. and Hellerstein, H. K. (156)

Author	Reference Number	Age of Group Studied yrs.	Non diabetics: No of Cases Studied	Non diabetics: Incidence of Coronary Thrombosis %	Diabetics: Incidence of Coronary Thrombosis %	Diabetics: No of Cases Studied
Enklewitz, M.	164	50–69	520	16	31	74
Root, H F and Sharkey, T P	159	40 and over	170	2	19 6	157
Root, H F et al	160	40 and over	2,310	8	35	326
Lisa, J R et al.	161	40 and over	2,250	22	30	193
Stearns, S. et al	162	Over 40	400	23	64	50

TABLE VIII

Coronary Arteriosclerosis—Occurrence in Diabetics and Non-diabetics at Postmortem—Liebow, I. M. and Hellerstein, H. K. (156)

Author	Reference Number	Degree of Sclerosis	Age Group yrs.	Non-diabetics: No of Cases Studied	Non-diabetics: Incidence of Coronary Sclerosis %	Diabetics: Incidence of Coronary Sclerosis %	Diabetics: No. of Cases Studied
Blotner, H.	157	Well Marked	Controls 40–80 Diabetics 13–85	450	21	45	77
Nathanson, M. H.	158	Marked	Over 50	249	8.2	52.7	74
Root, H. F. and Sharkey, T. P.	159	Occlusive	40 and over	170	13	46.7	157
Root, H. F. et al	160	Narrowing with or without occlusion	40 and over	2,310	29	56	316
Lisa, J. R. et al.	161	Severe	40 and over	2,250	29	46	193
Stearns, S. et al	162	Narrowing with occlusion or myocardial fibrosis	Over 40	400	37	74	50
Millard, E. B. and Root, H. F.	163	Occlusion or marked narrowing		2,310	29	68	106

almost invariably be atherosclerotic. Thus Warren found only four patients free of arteriosclerosis among 484 autopsied cases with diabetes for five years or longer (165). This "universality of atherosclerosis in diabetes" (166) is true for young and old, female and male.* Diabetes eliminates the relative immunity of women to atherogenesis (Table X) (599). It condemns the young to premature "degenerative" vascular disease (600–602).

Studies on plasma lipids in patients with diabetes have not yielded entirely consistent results (54). Most studies demonstrate the frequent occurrence of hyperlipemia in diabetics. In the pre-insulin era it was noted that elevation of plasma lipids was most frequent and marked in severe, long-standing diabetics who were prone to acidosis and ketosis (54).

Experiences in the insulin era have been less uniform. Some investigators assert that with adequate treatment of the defective carbohydrate metabolism, a normal plasma lipid pattern may be established and maintained in many diabetic patients (54, 167–170). Other workers fail to find any correlation between plasma lipid levels and degree of diabetes control (171).

Extensive work is only now beginning, correlating deranged plasma lipid patterns and atherogenesis in diabetic humans. A study of controlled diabetics by Man and Peters led them to conclude, "The level of serum cholesterol did not appear to be related to the severity of diabetes, the fat in the diet or the degree of arteriosclerosis" (167). Rabinowitch arrived at an almost opposite judgment: "Excess blood cholesterol is an important etiologic factor [in arteriosclerosis] in the young diabetic" (170). Recent studies tend to agree with Rabinowitch's conclusions (171–179). Thus the latest edition of Joslin's book stresses that the frequency

*Contrast, however, the rarity of atherosclerotic complications of diabetes in China (92) and Ceylon (587) (cf. page 22).

TABLE X

Incidence of Fatal Coronary Atherosclerosis in Diabetic and Non-Diabetic Males and Females—Postmortem Studies—Clawson, B. J. and Bell, E. T. (599)

Age Yrs.	Non Diabetic Male %*	Non Diabetic Female %	Diabetic Male %	Diabetic Female %
0-40	0 7	< 0 1	5.0	4.7
40-100	10	5 8	19 5	17.4

*% = per cent of all deaths attributable at postmortem examination to coronary atherosclerosis.

tionships among lipid metabolism, hypertension, arteriolonephrosclerosis and atherosclerosis in these people (cf. reference 608)? The data of Joslin and Wilson suggest that at least in diabetic children the renal lesion is the primary and chief problem (601). Obviously much additional work will be required to disentangle this apparently complex skein of causes and effects.

B. The Nephrotic Syndrome

Plasma cholesterol and the other lipids are markedly elevated in the nephrotic syndrome.* This hypercholesterolemia frequently occurs in cases of renal disease with edema and hypoproteinemia, "whether they have a true nephrotic syndrome or not" (184). Elevation of plasma lipid has not yet been pathogenically correlated with other features of the syndrome, e.g., edema, depressed basal met- [illegible] hypercholesterolemia, so that the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio is not altered. The frequent and striking participation of neutral fat in the hyperlipemia accounts for the marked lactescence of plasma in these patients (54, 184). Page and Farr were unable to influence this nephrotic hyperlipemia by varying dietary fat intake or by administering thyroid or choline (185). In their recent paper Gofman, *et al.*, report the ultracentrifuge identification in nephrosis of exceedingly high concentrations of the particular plasma lipoproteins (S_f 10–20 class) which they associate with atherogenesis (152).

*The nephrotic syndrome—"the combination of gross edema, hypoproteinemia, hypercholesterolemia, lipemia and heavy proteinuria, in the absence of congestive heart failure" (183)—occurs not only during the course of chronic diffuse glomerulonephritis, but also in renal amyloidosis, syphilis, intercapillary glomerulosclerosis and renal vein thrombosis.

and severity of arteriosclerosis in diabetes is correlated with long standing uncontrolled glycosuria and hyperglycemia, with concomitant hyperlipemia and hypercholesterolemia (178). Similarly, a recent study of 500 patients by Root and Wilson led them to conclude that premature atherogenesis and degree of diabetic control were intimately related (179). Further, Pomeranze and Kunkel found a close correlation between hyperlipemia and atherogenesis in 273 diabetic patients. Of the entire series, 50% exhibited elevated serum lipid levels, irrespective of diabetes control (171). Of those with severe atherosclerosis, 72% had hyperlipemia, with complete lipid fractionations revealing some lipid abnormality in 92%. Finally, the ultracentrifuge studies of Gofman, *et al.*, demonstrate that the plasmas of diabetic males and females frequently contain abnormally high concentrations of the particular cholesterol-containing lipoproteins (S_f 10–20 class) whose appearance they associate with atherogenesis (152). Certainly, therefore, considerable data are extant demonstrating an association between disturbed lipid metabolism and atherogenesis in human diabetes mellitus. If the correlations in diabetics are not always consistent, this probably reflects the complexity of this disease state in man and the present inadequacy of our knowledge (54, 155, 180–182). The difficulties involved are readily illustrated by focussing attention upon the renal vascular lesion in diabetes mellitus and the associated hypertension (600–606). We have already noted recent evidence indicating that lipids may be key factors in the pathogenesis of this lesion (cf. page 13) (568, 577–581). Further note should be taken of the observation made by Man, *et al.*, that plasma cholesterol levels tend to rise coincident with the development of clinical manifestations of intercapillary glomerulosclerosis (605). Is this a renal hyperlipemia (cf. pages 49, 59, 224, 249)? Why is it not consistently observed (cf. ref. 607)? Is the hypertension a renal hypertension? What are the rela-

Relatively few studies have been done on the incidence and severity of atherosclerosis in nephrotics. All students of the subject, however, agree that the prolonged hyperlipemia of the nephrotic syndrome is associated with intensified atherogenesis (4, 80, 183, 186–193). Hueper has reviewed much of the extant literature (4). He cites several authors who have described cases of juvenile nephrosis with moderate to extensive coronary and aorta atheromatosis at autopsy. Gofman reports a case of marked atheromatosis in a nephrotic child (152). Schwarz and Kohn record 9 autopsied cases of nephrosis in children. In their experience, "Atherosclerotic changes, though occasionally occurring in cases of short duration, are found more frequently in those of longer duration" (192). As in diabetes, therefore, the time factor appears to be important for atherogenesis in nephrotics.

Steiner and Domanski report lipid and autopsy findings in 54 young patients dying of chronic renal disease (193). The incidence of aorta and coronary atherosclerosis in these patients is indicated in Table XI, abstracted from this paper (193). In brief 96% of patients age 1–39 years dying of chronic glomerulonephritis had gross aorta atherosclerosis, 70% had gross coronary lesions. A comparable control group exhibited significantly less atherosclerosis (Table XI). In 17 of 30 patients coming to autopsy, Steiner and Domanski found serum cholesterol values exceeding 300 mg %, in 12 they were over 400 mg %. In another group of 18 living patients followed during a two year period, hypercholesterolemia was observed in all on one or another occasion (193).*

* [illegible]

disease, hypertension, plasma cholesterol and atherosclerosis

TABLE XI

Occurrence of Gross Aorta and Coronary Atherosclerosis in Patients Age 1–39 Years, Dying of Chronic Glomerulonephritis*—Steiner, A. and Domanski, B. (193)

	Number of Patients	% with Aorta Atherosclerosis	% with Coronary Atherosclerosis
Glomerulonephritics	54	96	70
Controls	54	35	19

*The glomerulonephritic and control groups are fully comparable with respect to age and sex.

berg (198), Rosenthal (199), Means (200), Bruger and Rosenkrantz (333), and Marine (533).

In the light of this not inconsiderable body of evidence, the contention would appear untenable that "the incidence of arteriosclerosis in patients with long-standing hypothyroidism . . . . is not remarkable" (201). Hypothyroidism, myxedema and cretinism may be reasonably added to the list of clinical entities wherein an association has been demonstrated between hypercholesterolemia and atherosclerosis.

D. Biliary Obstruction

In diseases of the liver, various alterations of lipid metabolism occur with associated derangements of plasma lipid patterns (54, 202). These reflect the critical role of the liver in lipid metabolism. It serves as a principal organ for cholesterol synthesis, turnover and excretion (via the bile), and is a key way-station in the hemato-entero-hepatic circulation of this sterol (54).

A discussion of the complex changes in plasma lipids occurring in hepatic diseases is beyond the scope of this monograph (cf. 54, 202). Our concern is only with those hepatic diseases associated with hypercholesterolemia. Both hypercholesterolemia and hyperlipemia rapidly supervene following obstruction of the common duct, from whatever cause. The alterations in plasma lipids persist in cases of prolonged obstruction, often maintaining themselves until the preterminal period of the disease. The pathogenesis of this hyperlipemia remains obscure (4, 54).

This hypercholesteremia of biliary obstruction is associated with a significant rise in the plasma $\frac{\text{free cholesterol}}{\text{total cholesterol}}$ ratio. Further, hyperphospholipemia occurs concomitantly with hypercholesteremia, so that the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$

In summary, the conclusion appears valid, on the basis of the available data, that the hypercholesterolemia of renal disease is associated with intensified atherogenesis.

C. Hypothyroidism

Numerous observers have repeatedly demonstrated that the plasma cholesterol and the other plasma lipids tend to be elevated *in hypothyroidism and myxedema* (54). Peters and Man report that the ratios of free to total cholesterol, and total cholesterol to lipid phosphorus are not affected by thyroid disease. *Similarly, the level of neutral fat in the* [illegible]

tients, found the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio to be increased, and the $\frac{\text{free cholesterol}}{\text{total cholesterol}}$ ratio to be decreased (195).

The altered plasma lipid pattern of hypothyroidism is associated with a low level of serum precipitable iodine, reflecting the deficiency of circulating thyroid hormone in *these patients* (196, 197). *Further, Gofman, et al., have* found appreciable concentrations of S_f 10–20 lipoprotein molecules in the plasma of hypothyroid patients. Administration *of thyroid hormone sometimes lowers their concentration* (152, 153, 567).

Hueper has reviewed much of the literature concerning *the increased incidence and severity of atherosclerosis, particularly* of the coronary arteries, in hypothyroidism and myxedema (4). He cites several workers who have recorded marked *atherosclerosis in cretins, among them Hoelzer's* case of a three-year-old girl with congenital athyreosis. Other reports of a confirmatory nature have emanated from Fish-

These findings led Ahrens and Kunkel to conclude that intensified atherogenesis is not a characteristic feature of biliary obstruction, despite attendant hypercholesterolemia. The authors related this absence of marked atherosclerosis to the normal ratios among the plasma lipids, and particularly to the normal C/P ratios. They regarded the interrelations among the plasma lipids as of greater significance for atherogenesis than the absolute level of cholesterol per se.

These data of Ahrens and Kunkel have been supplemented recently by the findings of Gofman and associates. They accomplished ultracentrifugal analyses of plasmas from cases of biliary obstruction with marked hyperlipemia. Unique plasma lipoprotein patterns were revealed (567). In some cases, S_f 10–20 cholesterol-bearing lipoprotein molecules were only slightly elevated, despite hypercholesteremias in excess of 1000 mg.%. Gofman predicts that such patients should be relatively free of atherosclerosis (567). In other patients, S_f 10–20 class molecules were present in appreciable amounts, although less than would be expected in view of the total cholesterol level. As in Ahrens and Kunkel's study, a larger series of cases and an attempt at biochemical-pathologic correlation are indicated here.

The findings of other investigators are not fully in accord with those of Ahrens and Kunkel concerning the incidence and severity of atherosclerosis in patients with chronic biliary obstruction. In addition to the reports cited by Hueper (4), several recent studies of both primary and secondary biliary obstruction are to be found in the literature (58, 206, 207). Significant atherosclerosis was frequently noted in these hyperlipemic patients. Hence the biochemical-pathological correlations noted by Ahrens and Kunkel would appear to require confirmation in larger series of cases. *In any event, the data available from all sources support a* tentative conclusion that the hypercholesterolemia of chronic biliary obstruction is associated with intensified

(C/P) ratio either remains normal or decreases (54, 203). The neutral fat is often altered only moderately, hence the plasma $\frac{\text{neutral fat}}{\text{lipid phosphorus}}$ ratio is low. As a consequence of this plasma lipid pattern, frank lactescence often is absent, despite severe hyperlipemia. This contrasts significantly with nephrosis and resembles *primary hypercholesteremic* xanthomatosis.

At present it is difficult to state definitely whether the hypercholesterolemia of biliary obstruction is associated with intensified atherogenesis. Rosenthal regarded the hyperlipemia of biliary obstruction as of too short duration to permit significant atherogenesis (80). However, not a few cases exhibit hyperlipemia of many months' duration. Hueper has reviewed the earlier literature on the concurrence of chronic jaundice and xanthomatosis (4). Since these reports deal chiefly with skin and tendon lesions, the incidence of atheroma is difficult to establish. However, atheromatous lesions were undoubtedly present in some of these patients

Extensive studies on the lipid disturbance in biliary obstruction have recently been reported by Ahrens and Kunkel (203–205). These investigators emphasized the significance for atherogenesis of the unique pattern of hyperlipemia frequently observed in this syndrome, particularly the predominance of phospholipid, the normal plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ (C/P) ratio and the absence of lactescence. Clinical evidence of coronary atherosclerosis was conspicuously absent in their 21 cases of long standing biliary obstruction with prolonged hyperlipemia. Seven of these patients came to autopsy, none with antemortem evidence of atherosclerosis.

tensive atherosclerosis. This latter group, unlike the former, had exhibited elevated plasma C/P ratios antemortem.

TABLE XII

Incidence of Hypercholesterolemia, Xanthomata and Coronary Atherosclerosis in 201 Members of Families with Primary Hypercholesterolemic Xanthomatosis—Adlersberg, D. *et al.* (223)

% with Hypercholesterolemia	% with 1 or more xanthomatous stigmata	% with coronary artery disease	% with complete syndrome*
69	59	40	24

*Complete syndrome is hypercholesterolemia, skin and tendon xanthomata, and coronary atherosclerosis

atherogenesis, at least in those cases exhibiting elevated plasma C/P ratios.

E. Essential (Familial) Hypercholesterolemic Xanthomatosis

In this familial disturbance, the plasma exhibits a lipid pattern characterized by an increase in total cholesterol, a slight rise in phospholipids, little or no alteration in neutral fat, a normal ratio of free to total cholesterol, and an elevated ratio of total cholesterol and free cholesterol to phospholipid (54, 58, 208). Since neutral fat is not increased, frank lipemia (creamy plasma) is not seen. The postabsorptive plasma is usually translucent, or on occasion somewhat opaque. Gofman and associates report markedly elevated levels of class S_f 10–20 lipoprotein molecules in such plasma (152, 556, 567).

Essential hypercholesterolemic xanthomatosis is apparently an endogenous disturbance of lipid metabolism. Thannhauser suggests that cholesterol production exceeds cholesterol excretion, and this imbalance is responsible for the altered plasma lipid pattern (58, 209, 210). This concept is hypothetical, since demonstrative experimental data are lacking.

The hypercholesterolemia of essential xanthomatosis is resistant to present methods of treatment. Efforts to lower the plasma cholesterol level by the administration of lipotropic factors have met with indifferent results (58, 208, 211). Some authors assert that low fat diets may effect considerable depression of the hypercholesterolemia; others report inconstant responses (58, 208, 212, 213). Keys recently obtained marked falls in plasma cholesterol concentration in cases of idiopathic hypercholesterolemia treated with the cholesterol-free, fat-free rice diet (119). Thyroid hormone has also been reported to reduce the hypercholesterolemia (58), although this has been denied recently (208).

Atheromatous involvement of the arteries is frequent in

50. Incidence of hypertension was low in these patients and cannot be considered a factor in atherogenesis (223).

Although we have cited the statistics of Adlersberg, Parets and Boas in some detail, similar data are present in the writings of other workers (214–224). These numerous reports demonstrate conclusively that the hypercholesterolemia of essential (familial) xanthomatosis is associated with a high incidence and early onset of vascular xanthomatosis in general and coronary atherosclerosis in particular.

F. Hypertension

The problem of the interrelationships among hypertension, lipid metabolism and atherosclerosis is one of the most complex in investigative medicine. A comprehensive review of the available data is beyond the scope of this monograph. The interested reader is referred to references 4, 10, 35, 54, 57, 80, 186, 187, 225–236. Present knowledge may be adequately summarized in a few sentences: Hypertension in man is typically associated with increased incidence and severity of atherosclerosis. Conversely many people with atherosclerosis have hypertension. However, long standing hypertension may be unaccompanied by atherosclerosis.* And atherosclerosis may develop in normotensive persons. Some investigators report that a hypercholesterolemic tendency is common among patients with hypertension (54). Others maintain that normocholesterolemia prevails in essential hypertension. They state that hypertensives exhibit hypercholesterolemia only when nephritis or another process is present to induce the rise in plasma cholesterol concentration (54). In view of contemporary unclarity concerning the pathogenesis of hypertension, and particularly the role of the kidneys and the adrenals in its causation, such contradictory findings and conflicting views are not surprising.

*Snapper reported that arteriosclerosis complicating hypertension is very rare in China (92) (cf. page 21).

persons with primary hypercholesterolemic xanthomatosis (4, 208, 214–224). Precise data on incidence of coronary atherosclerosis in patients with this metabolic disease have recently been collected by Adlersberg, Parets and Boas (223). These workers observed a series of 64 patients with xanthomatous stigmata. They were able to study the families of 35 of these patients. Data were accumulated on a total of 201 persons. Some of the findings in these 201 cases are summarized in Tables XII and XIII The high incidence of coronary atherosclerosis in these hypercholesterolemic persons is evident. In addition, these data demonstrate that the incidence of arterial disease directly parallels the degree of hypercholesterolemia (Table XIII). Further, analysis of the patients with coronary artery disease reveals that 86% of them had hypercholesterolemia (plasma cholesterol greater than 300 mg.%).* Finally, an early onset of coronary atherosclerosis was noted in this series Of the patients with cardiac symptoms and signs, 60% developed them before the age of

TABLE XIII

Plasma Cholesterol Levels and Incidence of Coronary Atherosclerosis in 201 Members of Families with Primary Hypercholesterolemic Xanthomatosis—Adlersberg, D. *et al.* (223)

Range of Plasma Cholesterol mg %	Incidence of Coronary Atherosclerosis %
> 300	45
> 400	53
> 600	67

*Bloor's oxidative method for cholesterol (116, 117).

lesterol, in patients receiving cortisone (240). Less consistent changes have been observed during ACTH administration (240, 241). The mechanisms of these pituitary-corticosteroid effects on cholesterolemia remain to be elucidated. Significant contributions to our knowledge of lipid metabolism and atherogenesis may be anticipated from clinical and laboratory studies of this problem.

H. Coronary Atherosclerosis

Our survey of diseases associated with sustained hypercholesterolemia demonstrated that atherosclerosis is a frequent byproduct of this disturbance in lipid metabolism, regardless of cause. A further clinical question remains to be posed: *Is a hypercholesterolemic or xanthomatous tendency present in patients with atherosclerosis?*

Until recently, this question was answered in the negative by most students of the subject. Thus Ophüls wrote in 1933, "One important difference between the condition in man and in rabbits is that it usually requires a very marked cholesterolemia to produce the characteristic changes in the rabbit, while in most cases of human arteriosclerosis it is not possible to demonstrate the presence of an excess of cholesterol in the blood during the course of the disease" (242).* In the same monograph Wells stated, "The evidence as to the relation of blood cholesterol to human arteriosclerosis or hypertension is conflicting" (73) In 1946, Peters and Van Slyke wrote, "No general disturbance of lipid metabolism has been demonstrated in patients with atherosclerosis" (54).

As Davis, Stern and Lesnick point out (231), the equivocal data in the older literature (cf. 186, 231) reflected lack of careful consideration of criteria for the diagnosis of

*Later (pages 135, 280) we shall deal in detail with this criticism of experimental work.

Further careful studies on cholesterolemia and lipid metabolism in various forms of human hypertension are certainly indicated. Along these lines, important data have recently been collected by Gofman and his associates (152, 153). They found that some hypertensives exhibit elevated plasma concentrations of S_f 10–20 lipoproteins, even in the presence of normocholesterolemia These changes in the S_f 10–20 class levels are not correlated with the degree or duration of hypertension. The significance of these alterations for atherogenesis in hypertensives remains to be definitively established However, Gofman, *et al.* further reported that hypertensive patients with coronary artery disease tend to have higher S_f 10–20 levels than do individuals with uncomplicated hypertension (538) (cf. page 106). These findings call to mind Aschoff's statement, "From plasma of low cholesterol content no deposition of lipoids will occur even though the mechanical conditions are favorable" (102).

G. Hyperadrenocorticism

Clinical states of hyperadrenocorticism, e.g., Cushing's syndrome, are associated with inordinately severe atherosclerosis (4, 237–239). While these diseases are rare, the recent introduction of adrenocorticotrophic hormone (ACTH) and corticosteroids (cortisone, *et al.*) into clinical therapeutics requires directing attention to the possibility that atherosclerosis may emerge as a complication of chronic treatment with these hormones

Humans with clinical hyperadrenocorticism exhibit no consistent abnormalities in plasma lipid patterns or concentrations (54). The plasma cholesterol level is more often elevated than reduced. Peters and Van Slyke attribute this finding to the diabetes and/or renal disease frequently accompanying hyperadrenocorticism (54). However, recent data bring this interpretation into question. Thus Adlersberg, *et al.*, report a rise in plasma lipids, including cho-

TABLE XIV

Plasma Total Cholesterol Levels in Patients with Coronary Atherosclerosis

Author	Reference Number	Normal Controls: Mean Plasma Total Cholesterol mg %	Coronary Cases: Mean Plasma Total Cholesterol mg %
Davis, D. et al.	231	218	260
Poindexter, C. A and Bruger, M.	243	195	248
Steiner, A. and Domanski, B.	244	254	355
Herrmann, G R.	245	193	254
Boas, E P. et al.	246	—	316
Steiner, A	9	254	355

atherosclerosis. Failure to distinguish between atherosclerosis and hypertension was particularly frequent.

A succession of significant clinical and experimental investigations compel at least a modification of the viewpoint cited above. In recent years several groups of workers have compared plasma cholesterol levels in normal persons and patients with coronary disease. Some of their findings are summarized in Table XIV. Other studies have yielded similar data (107–111, 148, 149, 247–253). Gertler, *et al.*, have recently published detailed findings on plasma lipids in coronary atherosclerosis (107–111). We have abstracted Table XV from one of their reports (107).

These data and others in the literature consistently demonstrate a higher mean plasma total cholesterol concentration in groups of people with coronary atherosclerosis than in control groups.* Moreover, atherosclerotic patients as a group also have higher plasma phospholipid and fatty acid concentrations and an elevated ratio of plasma total cholesterol to phospholipid. Thus the normal interrelationships among the plasma lipids are altered in the atherosclerotic group. These differences are particularly prominent if comparison is made between a group of atherosclerotics under 40 or 50 and a normal group of the same age (107–111, 246, 249, 250).

The data of Boas, *et al.*, further demonstrate that a hypercholesterolemic (xanthomatous) tendency is an important factor in atherogenesis, particularly in young persons (246). These investigators collected a consecutive series of 122 unselected patients with coronary artery disease beginning before age 50. Fifty-eight percent of these had a serum cholesterol concentration above 300 mg.%,† the average for

*Among recent reports, only the findings of Lande and Sperry do not support this conclusion (83). They could demonstrate no correlation between plasma cholesterol levels and either aorta grading for atherosclerosis or aorta cholesterol content.

†Normal range: 180–220 mg % (116, 117).

these 71 patients being 365 mg.%. Boas and his colleagues also evaluated the siblings of 50 of these patients. In 15 of these 50 families, all or most of the siblings had a plasma cholesterol level above 300 mg.%. In nine other families, one-half of the members available for study exhibited hypercholesterolemia. Many of these siblings gave evidence of xanthomatous stigmata, including coronary atherosclerosis.

A further problem merits consideration at this point—the problem of the increasing incidence of coronary atherosclerosis with age (cf. Table X, page 46) (599). Is the cho-

TABLE XVI

Changes in Cholesterol Level with Age—Keys, A. (254)

Age (yrs.)		Number	Cholesterol (mg %)	
Mean	Range		Mean	S. D.
19	17–20	109	173.2	± 30.2
22.5	21–25	92	179.5	± 36.7
32	27–35	17	198.2	± 25.3
43	39–44	16	222.4	± 25.4
46	45–47	87	242.5	± 37.5
49	48–50	98	249.1	± 42.8
52.5	51–55	102	251.9	± 43.0
68	63–74	20	233.9	± 37.4
All Ages		541	218.7	± 50.6

TABLE XV

Serum Cholesterol, Serum *Phospholipids, and* Cholesterol/Phospholipid Ratio in Control and *Coronary* Artery Disease Groups of *Patients—Gertler, M. M.* and Garn, S. M. (107)

	Serum Cholesterol mg %		Serum Phospholipids mg %		Ratio $\frac{\text{Cholesterol}}{\text{Phospholipids}} \times 100$	
	Control Group	Coronary Disease Group	Control Group	Coronary Disease Group	Control Group	Coronary Disease Group
Number of Patients	146	97	146	61	146	60
Range	148–332	167–490	215–415	195–414	52.0–104.4	60.4–110.8
Mean	224.4	286.5	299.3	316.4	75.1	89.4
Standard Deviation	42.6	64.9	40.2	52.2	10.9	15.9
Standard Error	3.5	6.6	3.3	6.6	.92	2.04

tion by 30 years of age is reached by the female during the 50 to 60 year decade" (556).

Jones, Gofman, et al., interpret the presence in plasma of lipoproteins of classes S_f 12 and above as a reflection of a lipid metabolic error significantly related to atherogenesis (cf. below, page 99) (556). Hence they regard as significant the correlation between rising S_f 12–20 levels with age and rising incidence of coronary atherosclerosis with age.

The foregoing data bring us to another key problem—the problem of the sex differential in incidence of coronary atherosclerosis. This sex differential is particularly marked in the younger age groups. Males under 40 years of age are victimized by coronary atherosclerosis 10–24 times more frequently than females (556, 599, 610). In the older age groups this differential becomes progressively less and is probably obliterated (cf. Table X, page 46) (599, 611–613). Is the cholesterol concept of atherogenesis consistent or inconsistent with these phenomena? Data on plasma lipids in men and women at different ages are presented in Tables XVII and XVIII (609, 614). It is evident that women have either essentially the same or higher plasma cholesterol levels than men. The plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ (C/P) ratios are not significantly different. These findings suggest that the sex differential in coronary atherogenesis is not attributable to differences in absolute concentration of plasma cholesterol and phospholipid. This suggestion is given additional support by data indicating a hypercholesterolemic tendency in pregnant women (54, 55).

However, the foregoing facts by no means exhaust this problem. Thus Eilert showed that estrogens tend to depress C/P ratios in menopausal women, principally by elevating plasma phospholipid (274) (cf. also, references 711, 712). Block, et al., found that, compared to women, both normal

lesterol concept of atherogenesis consistent or inconsistent with this phenomenon? Several recent studies afford data relevant to this question. Thus Gertler, *et al.* (110), Keys (254) and Kornerup (609) all found a tendency for the plasma cholesterol level to increase with age (Table XVI). This is true of a healthy group and of a coronary disease group (110). In the latter, the normal physiologic rise is superimposed upon the initial pathologic rise. Hence at every age level between the third and sixth decade the coronary disease group has markedly higher and more variable serum cholesterol levels than the healthy group (110).* The possibility presents itself that the tendency for the plasma cholesterol to rise with age is related to the increased incidence of atherosclerosis with age. This possibility receives further support from the data of Gofman, Jones and colleagues (556, 567). "Both young men and young women show similar patterns of lipoproteins and low levels of S_f 12–20 lipoproteins up to 25 years of age, although there has been some increase from the levels of the 0–15 year old group. The male population from 25 to 30 years undergoes a striking transformation which elevates the S_f 12–20 level from a median concentration of 28 mg.% to 39 mg.%. Beyond 30 years of age the clinically normal male population shows almost no further significant change even through the sixtieth year. The normal female does not show the striking change from 26 to 30 years of age that is seen in the male. The female population increases slowly and steadily in S_f 12–20 level over the entire 25 to 60 year age span. A level essentially identical with that attained by the male popula-

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[illegible]

other factors.

TABLE XVIII

Plasma Lipids of Normal Males and Females—Block, W. J. *et al.* (614)

Sex	Number of Persons	Age Yrs.	Plasma Total Cholesterol mg %	Plasma Lipid P* mg %	C/P
♂	23	27–46	181	9.0	20.1
♀	20	19–42	173	9.0	19.2

$^*\frac{\text{Phospholipid}}{26}$.

TABLE XVII

Plasma Lipids in Normal Males and Females at Different Ages—Kornerup, V. (609)

Sex	Age Yrs.	No of Persons	Total Cholesterol mg %	Lipid Phosphorus mg %	C/P
♂	1–14	51	201.3	7.5	26.8
♀	1–16	44	218.0	8.1	26.9
♂	19–46	54	203.0	6.6	30.8
♀	20–39	22	224.5	7.4	30.3
♂	50–82	26	237.3	7.4	32.1
♀	63–96	15	235.3	8.3	28.4

clusions of Jones, Gofman, et al., concerning the basis for the sex differential in incidence of coronary atherosclerosis. Nevertheless, their findings indicate that this sex differential may be explicable within the general framework of the cholesterol concept of atherogenesis.

At this point it may be necessary to re-emphasize the basic tenet of the cholesterol concept of atherogenesis, i.e., that atherogenesis is consequent upon an alteration in lipid metabolism. In approaching such problems as the age and sex differentials in incidence of coronary atherosclerosis, we posed the following question, based on the cholesterol concept: Are there age and/or sex differences in plasma lipid patterns? The presently available answers to this question would appear to be fruitful for our understanding of atherogenesis. Based on the cholesterol concept, several further questions related to the age-sex problem require elucidation, among them: What are the accumulated influences of various diets on lipid metabolism in the two sexes over the years (cf. page 32)? What changes occur in endocrine function with age in the two sexes, influencing lipid metabolism and atherogenesis (610, 615–623)? Are the relationships between age-sex and atherogenesis mediated via alterations in lipid metabolism which develop via the influence of age and/or sex on organs such as the liver, thyroid, adrenals, gonads, kidneys, pituitary? Or is the older concept valid, i e, of age and sex-influenced changes in the arterial wall—changes in its lipid metabolism, in its penetrability to lipids, in its ability to dispose of lipids? Under what conditions (of lipid metabolism, age, sex, etc.) are atherosclerotic lesions primary, i.e, developing in hitherto normal vessels—and under what conditions are they secondary to previous non-atheromatous senescent and/or morbid processes in the intima and media (cf. pages 76–86, 229, 251)?

We emphasize that these problems arise from a careful consideration of the full implications of the cholesterol con-

and atherosclerotic males develop a significantly greater lactescence of plasma after a fatty meal (614). As already indicated, Jones, Gofman and colleagues found significant sex differences in levels of class S_f 12–20 plasma lipoproteins during the fourth, fifth and sixth decades of life (556). These differences, they maintain, are the basis for the sex differential *in susceptibility to coronary atherosclerosis.* "This is adequate explanation for the fact that the male under 40 years of age shows approximately a twentyfold greater occurrence rate of myocardial infarction than does the female of the same age. Assuming a level of 60 mg.% S_f 12–20 as prognostic of rather active atherosclerogenesis, and comparing the males and females at 31 to 40 years of age, the female has only 2% of all its normals above 60 mg.%, whereas the male population of the same age has 20% of its members above this same level. This tenfold difference in numbers above 60 mg.% is in good agreement with the approximately twentyfold difference in occurrence rate of myocardial infarction at this age. At 40 years of age 8% of females are above 60 mg.% while 22% of males are above this value at this age. The normal female has shown a rise from 3% above 60 mg.% at average age 35 years to 28% above 60 mg % at average age 55 years. This ninefold increase in the percent of the female population above 60 mg.% is consistent with the marked advance in frequency of clinical sequelae of atherosclerosis in the 50 to 70 year age category. The female at 50 to 60 years of age is essentially equivalent to the male of that age in S_f 12–20 level, or in atherosclerogenesis, so that each year that passes reduces the relative protection of her relatively lower atherosclerogenicity of earlier years. Thus it is not surprising that the ratio of clinical sequelae of atherosclerosis drops from approximately 20:1 below age 40 to approximately 1:1 above age 60 years for males compared with females" (556). Only further work will determine the final validity of the bold con-

clusions of Jones, Gofman, et al, concerning the basis for the sex differential in incidence of coronary atherosclerosis. Nevertheless, their findings indicate that this sex differential may be explicable within the general framework of the cholesterol concept of atherogenesis.

At this point it may be necessary to re-emphasize the basic tenet of the cholesterol concept of atherogenesis, i.e., that atherogenesis is consequent upon an alteration in lipid metabolism. In approaching such problems as the age and sex differentials in incidence of coronary atherosclerosis, we posed the following question, based on the cholesterol concept: Are there age and/or sex differences in plasma lipid patterns? The presently available answers to this question would appear to be fruitful for our understanding of atherogenesis. Based on the cholesterol concept, several further questions related to the age-sex problem require elucidation, among them: What are the accumulated influences of various diets on lipid metabolism in the two sexes over the years (cf. page 32)? What changes occur in endocrine function with age in the two sexes, influencing lipid metabolism and atherogenesis (610, 615–623)? Are the relationships between age-sex and atherogenesis mediated via alterations in lipid metabolism which develop via the influence of age and/or sex on organs such as the liver, thyroid, adrenals, gonads, kidneys, pituitary? Or is the older concept valid, i e., of age and sex-influenced changes in the arterial wall—changes in its lipid metabolism, in its penetrability to lipids, in its ability to dispose of lipids? Under what conditions (of lipid metabolism, age, sex, etc.) are atherosclerotic lesions primary, i e., developing in hitherto normal vessels—and under what conditions are they secondary to previous non-atheromatous senescent and/or morbid processes in the intima and media (cf. pages 76–86, 229, 251)?

We emphasize that these problems arise from a careful consideration of the full implications of the cholesterol con-

cept of atherogenesis. The answers obtained to these questions will be, in time, further tests of the validity of this concept. Certainly clinical and laboratory investigation of these problems may yield fruitful insights, hitherto concealed, into the atherogenic process.

While our survey of the relationships between lipid metabolism and coronary atherosclerosis has led to the posing of a number of unsolved problems, several facts remain clear and definitive. In particular, it is evident that people with coronary atherosclerosis (as a group) exhibit a hypercholesterolemic tendency and other unusual lipid metabolic patterns (C/P ratios, S_f 12–20 levels, etc.). Unfortunately, studies attempting similar correlations between cholesterolemia and atherosclerosis of other arterial trunks supplying major organs have not been attempted. Despite the high morbidity and mortality due to cerebral atherosclerosis, for example, investigations of the pathogenic relationship of this lesion to cholesterolemia are almost nonexistent, insofar as we have been able to ascertain (332, 554, 555, 567, 576, 586, 600–602, 612, 624–626). Both clinical and experimental data suggest the conclusion that atherogenesis proceeds according to different laws in different arterial beds (cf. below, page 133). Hence research along this line is sorely needed. These gaps in our knowledge, however, cannot controvert the significance of the demonstration of an association between cholesterolemia and coronary artery atherosclerosis.

Definitive though this conclusion is, its formulation does not fully resolve the problem at hand. Certain facts still remain to be accounted for. Thus, perusal of Table XV quickly reveals that some people with coronary atherosclerosis definitely have apparently normal plasma cholesterol concentrations. What is the relationship between cholesterolemia and atherogenesis in such persons? Gubner and Ungerleider have presented data bearing upon this problem.

TABLE XIX

Incidence of Aorta Atherosclerosis in Mildly Hypercholesterolemic, Normocholesterolemic and Hypocholesterolemic Persons*—Gubner, R. and Ungerleider, H. (57)

	Mean Plasma Total Cholesterol mg %	Incidence of Aorta Atherosclerosis %
Mild Hypercholesterolemics	300	22
Normocholesterolemics	211	20
Hypocholesterolemics	160	7

*The three groups were equated with respect to age, weight and presumably sex and coexistent diseases.

They studied the incidence of aorta atherosclerosis in mildly hypercholesterolemic, normocholesterolemic and hypocholesterolemic individuals (57). The basis for their division into groups was the generally accepted range for normal plasma cholesterol concentration (54). Their data are summarized in Table XIX. Incidence of atherosclerosis was approximately identical in people with slightly elevated and presumably "normal" plasma cholesterol levels. In contrast, the hypocholesterolemic subjects exhibited a significantly lower incidence. These findings lead the authors to suggest that our standards for "normal" cholesterol may be scaled too high. Certainly at this stage of our knowledge this is a valid possibility. However, the question remains: Why did even 7% of the hypocholesterolemic group develop atherosclerosis?

A number of attempts have been made to account for atherogenesis in such individuals. Some investigators suggest that people who are now normocholesterolemic may have developed atherosclerosis during previous abnormal episodes of hypercholesterolemia. This eventuality cannot be ruled out, even though the plasma cholesterol concentration is ordinarily quite stable in a given individual over long periods of time (54). As Hueper stated, "The determination of the blood cholesterol content . . . merely renders information about the momentary condition of the blood, which may be entirely unrelated to the status of the blood prevailing when the vascular lesions were produced" (4). In support of this possibility, Steiner called attention to the fluctuating hypercholesterolemia frequently observed during postinfectious convalescent periods. He suggested, "It is conceivable that such a period of transient hypercholesterolemia might lead to deposits of cholesterol in the arteries" (56). These ideas are admittedly only hypotheses, without foundation in supportive data.

III ATHEROSCLEROSIS AND THE PLASMA LIPOPROTEIN COLLOIDAL COMPLEX (THE STATE OF AGGREGATION OF CHOLESTEROL IN PLASMA)

RECENTLY THE PROBLEM OF CHOLESTEROLEMIA AND atherogenesis has been approached from another aspect. Instead of focussing attention upon the absolute level of plasma cholesterol as the key variable, several workers studied the possible significance of various alterations in the state of aggregation of the plasma lipids. They emphasized that the quantitative determination of plasma cholesterol yields a datum highly incomplete with respect to cholesterol itself. Water-insoluble, hydrophobic cholesterol exists in a state of colloidal solution in biological systems. The colloidal cholesterol-containing aggregates (micelles) are complex structures with respect to size, configuration and composition. Their essential constituents are not only the other plasma lipids (phospholipids, fatty acids), but also proteins (4, 53–55, 57, 63, 64, 67, 86, 152, 204, 236, 255–257). These lipid and protein components of the plasma micelle are bound together in relatively fixed proportions. The colloidal bonds, whereby these large-sized lipoprotein moieties are built up, serve to bring cholesterol into plasma solution. The integrity of these bonds is decisive for maintaining plasma lipid stability.

Presumably, qualitative alterations in the plasma cholesterol-containing micelles may occur with little or no [illegible]

genesis.

These concepts are not new among investigators of the atherosclerosis problem. Almost inevitably, once a key place was assigned to cholesterol in the atherogenic process, such hypotheses had to be formulated to account for atherosclerosis in normo- and hypocholesterolemic persons. Several such explanations have been advanced.

CHOLESTEROL "SOLUBILITY"

Eck and Desbordes maintained that many patients with atherosclerosis have normal plasma cholesterol levels because their plasma is unable to hold a greater concentration of sterol in "solution." Additional cholesterol is precipitated in the blood stream, inducing atherogenesis (258, 259). They further claimed that the plasma solubility of cholesterol decreases with age. To this they attributed the increasing incidence and severity of atherosclerosis in the latter decades of life.

MACROMOLECULAR COLLOIDS

In 1941–1942, Hueper demonstrated that intravenous and intraperitoneal injections of macromolecular colloids (polyvinyl alcohol, methylcellulose, pectin, acacia) induced atheroma-like lesions in the large arteries of the dog. The foam cells in the intima in these cases contained the administered colloid, and not cholesterol (260–264). Based on these experiments, Hueper put forward the "anoxemia theory" of atherogenesis. "*The atheromatous process is* initiated by an imbalance of the plasmatic colloid equilibrium causing the precipitation of a cholesterol film on the intima. Such a film . . . interferes with the proper exchange of gases and nutritive substances between the blood and the vascular wall . . . The resultant injury to the endothelial cells causes increased permeability of the endothelial

lining and proliferation of the endothelial cells which take up cholesterol and are thereby transformed into foam cells. A similar conversion into foam cells takes place in the intima where cholesterol contained in the plasmatic fluid penetrating into the subendothelial space is phagocytosed by histiocytes. The instability of the plasmatic colloidal solution of cholesterol which starts this chain of events in the vascular wall may be due to quantitative imbalance in the cholesterol content (hypercholesterolemia), or it may result from an increase of substances in the plasma which decrease the dispersion of cholesterol and therefore favor its precipitation or from a decrease of substances which enhance the dispersion of cholesterol" (4).

In 1943, Hirsch and Weinhouse similarly suggested that an increase in the particle size of the plasma lipoprotein aggregates leads to phagocytosis by intimal cells. "This may be considered to be the initial stage of atherosclerosis" (86). This concept Hirsch and Weinhouse supported with experimental data demonstrating that emulsified cholesterol oleate injected intravenously into rabbits is removed from the plasma in a matter of minutes by tissue phagocytes. These investigators suggested that ". . . the phagocytic reactions observed in the intima occur as a response to the presence of coarsely dispersed lipids in varying stages of agglomeration" (86). They attempted to account for atherosclerosis in the absence of sustained hyperlipemia by making a further distinction between plasma and tissue factors: "The primary cause of the precipitation of lipid in cholesterol-fed rabbits is probably the hyperlipemia; in human atherosclerosis it is mainly a tissue factor increased under conditions of hyperlipemia" (86).

More recently Pollak (265) and Bevans (56) demonstrated that intravenous injections of artificially prepared cholesterol emulsions rapidly induce atherogenesis. "As early as three hours after the first injection of 0.5 gm. of choles-

terol, lipid is visible in the intima of the aorta. It first appears within the endothelial cells, which are swollen and prominent. At the end of 24 hours more lipid is visible in the endothelial cells and has spread to the intercellular substance. No intimal proliferation has occurred. At 72 hours less lipid is present in the intima but cellular proliferation is evident" (56). Recently Pollak and Wadler reported extensive studies on this type of arterial lesion, induced in animals by intravascular injection of cholesterol sols (627–633). Further, Bragdon demonstrated that this lesion developed not only when artificial lipid emulsions are injected, but also following intravenous administration to normal rabbits of hypercholesterolemic plasma from cholesterol-fed rabbits (634, 635). (Also, cf. Hueper's work, references 4, 260–264) These findings strongly support the concept that phagocytosis by the intima of larger lipid aggregates is a key initial process in atherogenesis.

In interpreting their data on plasma macromolecular colloids and atherogenesis, most of the foregoing workers developed hypotheses concerning a further problem: *How does lipid enter the arterial wall in the process of atherogenesis?* Numerous answers to this question have been advanced. Hueper (4), Leary (7), Katz and Dauber (38) and others have reviewed the status of this important problem. As already indicated, Virchow originally suggested that atherosis represents a fatty imbibition and metamorphosis secondary to inflammatory necrobiotic processes in the connective tissue cells and ground substance of the intima (75). This necrobiotic theory, which regards lipids in atherosclerotic lesions as mere secondary byproducts of previous pathological processes, was espoused by several workers (cf. 4). Other investigators in one form or another advanced the concept that the arterial intima imbibes cholesterol and lipids from the plasma, this process being intensified by hypercholesterolemia. Some, like Aschoff (102), Hirsch and

Weinhouse (86), and others (cf. 4), believe that age- or disease-induced alterations in the intima or media are essential prerequisites for precipitation and fixation of imbibed lipid. Thus Faber emphasized the importance of changes in the aorta ground substance occurring with age (636). He found that over the years the aorta develops an increased content of metachromatically stainable carbohydrate-sulfuric acid esters. These changes, he maintained, play a significant role in the deposition of cholesterol and the development of atherosclerotic plaques (636). Similarly, Dock advanced the concept that progressive fibrous thickening of the intima occurring with age favors atherogenesis, particularly in the coronary arteries (560, 568, 637).* With respect to this concept, Dock himself noted that marked intimal thickening may occur without atherosclerosis developing, *vice versa*, atherogenesis may proceed in certain arteries (e.g. renal) having practically no intima (568, 58; also cf. 12). Moreover, as already noted (cf. page 13), it is possible that diffuse concentric intimal thickening is itself a reaction to lipid deposition (577, 638). Lansing, *et al.*, presented another variation of the concept that previous age- or disease-induced change in arteries is an essential prerequisite for atherogenesis (569, 639–643). They stressed the importance of medial alterations (elastic tissue changes and calcification), as well as intimal fibrosis Several inconsistencies in this concept were indicated by discussants of Lansing's paper at the 1951 (Fifth) Conference on Factors Regulating Blood Pressure (569). In essence, Duff advanced a generally similar concept, stressing the importance of previous alteration of the artery for atherogenesis. He suggested in addition that hypercholesterolemia *per se* may be the factor responsible for the primary vascular wall injury (267). Hueper's anoxemia

*Dock no longer definitively adheres to his previous viewpoint that the sex differential in incidence of coronary atherosclerosis is due to a sex difference in thickness of the coronary intima (560, 568, 637).

theory is another variation on these themes. As indicated in the quotation on page 76, he suggested that colloidal cholesterol films precipitated from dysbalanced plasma coat the intima and damage it. The intima becomes increasingly permeable to lipids. The additional concept is advanced that intimal cells become phagocytic, take up cholesterol and undergo metaplasia into foam cells (4). Their experiments also lead Pollak (265) and Bevans (56) to adopt the hypothesis that intimal cells have lipid phagocytic capacities. In contrast to this view, Leary stated that foam cells are lipophages derived from reticulo-endothelial tissues in the liver, adrenal, lung, spleen, etc. These are desquamated into the blood stream and carried to the large vessels. They are specifically attracted by the arterial intima, which they penetrate to form foam cell cushions or atheromata—the initial lesions of atherosclerosis (7) (cf. page 84). The foam cells then break down, releasing their lipid contents. Cholesterol and the other lipids are phagocytosed by fibroblasts of the arterial wall. Youthful arterial tissue is able to dispose of this lipid. This capacity is diminished or lost with age. Cholesterol esters then accumulate and initiate the secondary reactive and degenerative processes leading to fully developed atherosclerotic plaques (7).

Like Leary, Anitschkow held that hypercholesterolemia can primarily induce atherosclerosis without any pre-existent alteration in the arterial wall (268). Unlike Leary, however, Anitschkow believed that the lipids entered the intima via an extracellular route, by direct filtration from the plasma. While maintaining that atherogenesis was possibly based on hypercholesterolemia alone, without vascular damage, Anitschkow also put forward a "combined theory": Previous damage to the arterial wall facilitated its penetration by lipids, with resultant superimposition of atherosclerosis on the original non-atheromatous lesion (268).

Winternitz, et al., developed a fundamentally different

concept of the pathogenesis of atherosclerosis (269). In the course of studies on the blood supply of the arterial wall, they described an intramural intimal circulation anastomosing with the adventitial vasa. These intimal vasa are most numerous at the origins of branching arteries. They generally increase in number with age. Moreover, they are always found in association with intimal atherosclerotic lesions. Winternitz, *et al.*, regarded this association as more than fortuitous They further observed frequent intramural hemorrhages from these vasa. Based on these findings, they advanced the hypothesis that such hemorrhages constitute the initial phase of atherogenesis. Disintegration of the red corpuscles at these hemorrhagic sites releases contained cholesterol and lipids. Atherogenesis proceeds from there (269).

Recently Duguid advanced a theory of atherogenesis akin to that of Winternitz, *et al* (644, 645). This concept is a resurrection of Rokitansky's thrombosis hypothesis (646). It maintains that the lesions of atherosclerosis are not infrequently the result of organization of arterial thrombi, including fibrinous deposits on the intimal surface (645). Foci of atheroma appear secondarily, as a result of fatty degeneration in the deeper zones of large thrombi (645). In accord with Duguid's views, Heard interpreted his histologic observations of aorta and renal atherosclerotic plaques as affording additional evidence that mural thrombosis is often an important factor in the pathogenesis of atherosclerotic lesions (647). Geiringer's studies also led him to favor this concept of atherogenesis (648) Like Winternitz, he noted the development of intimal vascularization as the aorta and coronary intimas thicken with age This intimal vascularization occurs by extension from the adventitial vasa vasorum and/or by retention of the vascular network of organizing mural thrombi. Interference with this intimal blood supply results in ischemic necrosis. This necrosis is responsible for

most of the clinical effects of atherosclerosis, according to Geiringer (648).

In an early review of the atherosclerosis problem, Katz and Dauber advanced a hypothesis combining some of the evidence of Leary and Winternitz with their own (38). "Our observation of clusters of foam cells within intimal vasa has indicated to us the importance of both the intimal blood supply and the importance of cholesterol-bearing foam cells. The localization of foam cells is determined by the presence of intimal vasa, their number and distribution. In the coronary arteries . . . [and] at points of branching the presence of intimal vasa permits the development of lesions. Any vascularized scar of the arterial wall caused by arteritis (syphilitic, rheumatic, etc.) would provide the vasa for localization of lesions . . . This indicates that not only the blood supply but the simultaneous presence of cholesterol-bearing foam cells is necessary for lesions to occur . . . The origin of the foam cells in experimental cholesterol atherosclerosis appears to be in the reticulo-endothelial cells of the liver and perhaps other organs. Their origin in man remains to be determined" (38).

In addition to emphasizing one or another among the foregoing factors in atherogenesis, most investigators recognize the role of local and systemic mechanical factors. Level of arterial pressure; presence of eddy currents and turbulence; and mobility, stretch and recoil of the vessel wall have all been implicated in atherogenesis, particularly in the focal pattern of location of lesions. A review of the extensive literature on these aspects of atherosclerosis is beyond the scope of this monograph. The reader is referred to discussions of these problems by Katz and Dauber (38) and other authors (4, 7, 11, 56, 57, 80, 186, 267–269).

Obviously, a host of different answers have been advanced to the question: *How does lipid enter the arterial wall in the process of atherogenesis?* In fact, when one attempts a sum-

mary view, the multiplicity of clashing hypotheses and theories is the most prominent feature. In assessing this situation, emphasis on a point of methodology may be of value. Practically without exception, all of the foregoing hypotheses and theories were derived from analysis of histologic data. Frequently investigation was confined to microscopic study of human autopsy material. When experiments were undertaken, they rarely went beyond routine histologic study of organs from cholesterol-fed animals. One conclusion would appear valid from our examination of the several theories derived in this way: Their very divergency highlights the probability that the methodology of morphologic pathology is per se inadequate to solve the problem at hand. If this conclusion is correct, the recent emphasis on greater experimental endeavor and less "comprehensive" speculation is indeed healthy. This trend merits every encouragement. For our part, we espouse no detailed "theory" of the pathogenesis of atherosclerosis. Together with most contemporary investigators, we base our work on the cholesterol concept of atherogenesis. It is within this general framework of reference that a detailed, patient, persistent research assault is proceeding against the host of unsolved problems concerning atherosclerosis

In this spirit, several workers have recently begun to tackle the difficult task of obtaining an experimental answer to the problem: How does lipid enter the arterial wall in the course of atherogenesis? Thus, based on Duguid's concepts, Harrison gave rabbits repeated intravenous injections of finely fragmented fibrin clot (649).* Multiple small pulmonary emboli resulted. These lesions were rapidly organized and the lumina of the vessels reconstituted. The intima of these vessels exhibited fibroblastic thickening. In one animal of the series, anisotropic lipid was demonstrable

*Cf the work of Bevans (281, 282), Bragdon (634, 635), Hueper (4, 260–264), Pollak (265, 627–633).

in a patch of intimal thickening. Based on these experiments, Harrison suggested that some cases of primary pulmonary arteriosclerosis may be the result of healed pulmonary embolism (649) (cf. ref. 659). Wartman also attempted to submit the concepts of Winternitz and of Duguid to experimental verification (650). He injected homologous blood into the arterial media of dogs. This blood disappeared within two months; histologically complete healing supervened or a fibrotic medial scar developed. No specimen exhibited atheromatous change in the overlying intima (650). Thus Wartman's studies did not yield experimental evidence supporting the theories of either Winternitz or Duguid.

Leary's concept of the origin of foam cells has also been put to test in recent experiments. Thus Simonton and Gofman labeled the reticulo-endothelial system of rabbits with radioactive materials (535). The animals were then fed cholesterol. After several months, the viability of the labeled cells was verified by demonstrating their continued ability to phagocytose India ink. Although atherogenesis occurred, with formation of intimal foam cell cushions, none of the lipophages in the lesions contained radioactive material (535). This finding indicates that—contrary to Leary's views—foam cells in atherosclerotic plaques are not derived from the phagocytic cells of the reticulo-endothelial system in liver, spleen, bone marrow, etc. Studies by Duff and Macmillan on the accumulation of colloidal thorium dioxide in lesions of experimental cholesterol atherosclerosis also yielded evidence failing to support Leary's concepts on the origin of lipophages (536). An additional significant fact emerged from the tracer studies of Simonton and Gofman (535), and of Biggs and Kritchevsky (653), viz., that exogenous cholesterol forms the bulk of the cholesterol in atherosclerotic lesions of cholesterol-fed rabbits. It would appear that cholesterol synthesized endogenously from small

molecules by arterial tissue (654) is probably not a significant factor in atherogenesis.

Hirsch and Weinhouse—utilizing biochemical analytical methods—compared lipid levels and lipid fractions in plasma and intima of normal and cholesterol-fed rabbits (85, 86). The intimal lipids had the same composition as the plasma lipids. The authors therefore concluded that ". . . the lipid deposits in atherosclerosis result from a non-selective deposition of the plasma lipids" (86). These findings tend to support the concept that the lipids of atherosclerotic lesions are derived from the plasma by a process of trans-intimal filtration. Recently Wilens also attempted an experimental evaluation of Anitschkow's infiltration theory (651). He excised normal human arteries and, utilizing various pressures (45–320 mm Hg.), distended them for 24 hours or more with normal human blood serum. Diffusion of serum through the arterial wall was demonstrated by chemical methods. Some cholesterol entered the arterial wall and was retained within it. Sudan IV staining confirmed this lipid deposition in the arterial intima. Wilens suggested that these experiments may reflect in some measure what occurs physiologically. Hence he concluded that they constitute ". . . direct and substantial evidence in favor of the filtration theory of lipid deposition in atherosclerosis" (651). The experiments of Evans, *et al.*, lend further support to this conclusion (652). They pulsated oxalated blood at various pressures against normal fresh strips of human aorta. When blood was obtained from cases with myocardial infarction or vascular disease, particulate deposits of birefringent lipid were demonstrable in the aorta wall (652). Use of blood from normal young adults resulted in little or no lipid deposition. Thus a significantly different result was obtained with blood from normal vs atherosclerotic individuals (652).

This observation brings us back to our point of departure in this discussion. From the incomplete evidence available,

it would appear that trans-intimal filtration from plasma may be the mechanism whereby lipids (lipoprotein complexes) enter the arterial wall in atherogenesis.* If this is so, the state of aggregation of cholesterol in plasma must be a key factor (together with the state of the intima) influencing the extent and rate of transudation of lipid into the arterial wall. Hence the importance of recent work on plasma cholesterol-bearing colloidal micelles.

ALIMENTARY HYPER- AND MACRO-CHYLOMICRONEMIA

Moreton recently advanced another variant of the concept that the state of aggregation of the plasma lipids is a decisive factor in atherogenesis (270–272). Unlike other investigators, he stressed the role of postprandial neutral fat hyperlipemia in atherogenesis.

In normal postabsorptive plasma, lipid aggregates (chylomicrons) are few in number and measure only ½–2 μ in size (54); the plasma is a clear limpid solution. The presence of hydrophilic phospholipids and proteins is essential for the maintenance of this small particle size and stability of the lipoprotein micelle.

Physiologically, both the number and size of these circulating lipoprotein aggregates increase in postprandial periods. Hyper- and macro-chylomicronemia, and gross lipemia (milky plasma) supervene. Chemical analysis of such postprandial plasma reveals a marked increase in neutral fat, with no change in cholesterol or phospholipid concentration (54). This type of hyperlipemia without hypercholesterolemia (as distinguished from the hypercholesterolemic hyperlipemia in hypothyroidism, diabetes mellitus, essential

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hypercholesteremic xanthomatosis, nephrosis, obstructive jaundice) (Fig. 6) occurs clinically in idiopathic familial hyperlipemia and in Von Gierke's disease (54). According to Moreton, "The cumulative effect of many fatty meals over a lifetime, by producing these transient showers of large lipid particles in the plasma, may be the underlying cause of the intimal lipid deposition in human atherosclerosis. . . . The increased *particle size of the lipids in* sustained or alimentary hyperlipemia is the stimulus to the phagocytosis in the intima by macrophages and the formation of the typical 'foam cells'" (270). ". . . . In normal

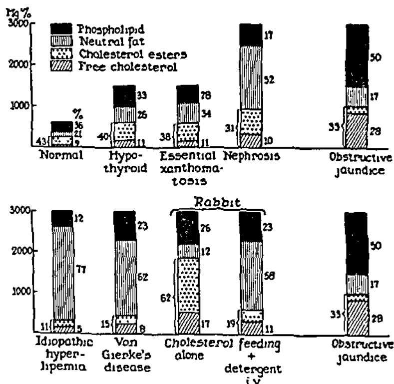


Fig 6. Typical serum lipid patterns in a variety of conditions associated with elevated serum lipids—Ahrens, E. H, *Jr.* (205).

humans deposition of lipid and atheromagenesis occur only during, and as a result of, the chylomicronemia produced by the ingestion of a fat-rich meal. . . . The primary factor and *sine qua non* in atherosclerosis is the presence in the *circulating blood of coarsely suspended colloidal particles* considerably larger than those found in normal plasma and composed of, or containing, a substance (cholesterol) relatively resistant to the resorptive and removal mechanisms of the arterial intima (macrophages and tissue fluid enzymes)" (271).

Becker, Meyer and Necheles recently supported this concept. They demonstrated that alimentary chylomicronemia is of significantly greater duration and intensity in people over 50, and suggested that this may account for the increased incidence and severity of atherosclerosis in older people (273). Further, Zinn and Griffith found a significantly greater ratio of macro-chylomicrons to micro-chylomicrons (lipomicrons) in the fasting blood sera of atherosclerotic patients, as compared with control subjects (655). Block, *et al.*, observed that following a standard fat meal men develop a significantly greater plasma lactescence than women of the same age group (614). In accord with Moreton's views, they suggested that this may be of significance for atherogenesis

Moreton's hypothesis hinges upon the idea that the altered state of dispersion of plasma lipids during alimentary hyperlipemia involves cholesterol. He postulates a qualitative alteration involving an increase in the size of cholesterol-containing micelles. Alteration in plasma total cholesterol concentration is not essential for atherogenesis, according to this concept. Moreton claims that biochemical analysis demonstrates cholesterol in macrochylomicrons separated from plasma by high speed centrifugation (270). However, contrary data were recently obtained by Gofman, *et al.* (152). These workers studied the entire spectrum of plasma lipids in

hyperlipemic man. They succeeded in carefully separating macrochylomicrons from other lipoprotein moieties by ultracentrifugation. Analysis of these macrochylomicrons revealed them to contain 5% or less cholesterol, a concentration inordinately low, in relation to Moreton's concepts. Moreover, before Moreton's ideas can be regarded as more than hypotheses, he must account for one clinical phenomenon fundamentally contradictory to his concept: In marked contrast to people with hypercholesterolemic hyperlipemias, patients with normocholesterolemic neutral fat hyperlipemias (e.g, idiopathic familial hyperlipemia, Von Gierke's glycogen storage disease) exhibit no unusual susceptibility to atherosclerosis (58, 205). In advancing his hypothesis, Moreton compared alimentary hyperlipemia with sustained hyperlipemia. An examination of the table he published (271) indicates that he failed to distinguish between the two types of sustained hyperlipemia, namely, normocholesterolemic and hypercholesterolemic. Instead, he equated both; or rather he equated hypercholesterolemic hyperlipemia and alimentary hyperlipemia The validity of this reasoning remains to be demonstrated experimentally—and with it Moreton's entire hypothesis.

TOTAL CHOLESTEROL/LIPID PHOSPHORUS RATIO

As we have already noted (cf. p. 54), Ahrens and Kunkel have recently emphasized another variant of the thesis that atherogenesis is due to abnormal interrelations among the plasma lipids and pathologic states of aggregation of the plasma lipoprotein micelles (203–205) Based on their studies of biliary obstruction, these investigators stress the significance of a disturbance in the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio.

Peters and Man were among the first to note that nor-

mally the plasma $\frac{\textit{total cholesterol}}{\text{lipid phosphorus}}$ (C/P) ratio is more constant than the concentration of either individual lipid (54, 147). The concept was advanced, "A proper balance between the lipids appears to be more sedulously protected and is, therefore, presumably more important than the absolute concentration of any one or all of the lipid components" (54). Attention was also called to the fact that the plasma $\frac{\text{total cholesterol}}{\textit{lipid phosphorus}}$ ratio was not a constant, either in normal or diseased people. Rather, it varied with the concentration of cholesterol. At higher plasma cholesterol levels, the ratio was higher, i.e., total cholesterol usually rose more rapidly than phospholipid, as the two increased (Fig. 7). Wilkinson recently demonstrated essentially the same correlation between free cholesterol and phospholipid (208), an expected finding in view of the relative constancy of the plasma $\frac{\text{free cholesterol}}{\text{total cholesterol}}$ ratio.

Neither Peters and colleagues nor Wilkinson correlated *these plasma lipid patterns and atherosclerosis.* The former deny that such correlations are valid (54). Hirsch and Weinhouse were among the first to emphasize that interrelations among the plasma lipids, rather than the absolute level of plasma cholesterol per se, might be significant in atherogenesis (86). Only lately, however, has attention been focussed particularly upon the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio.

In addition to the studies of Ahrens and Kunkel, the recent report of Eilert furnished information concerning the possible relationship of plasma C/P ratios to atherogenesis (274). She analyzed the effects of estrogens on plasma lipid fractions of female patients. Hormone administration usually effected a rise in serum lipid phosphorus and

a fall in total cholesterol. Consequently the plasma C/P ratio fell. The author noted that normal C/P ratios presumably protect against atherogenesis. The decreased susceptibility of pre-climacteric women to coronary atherosclerosis (227, 228, 275–277) might be attributable to the effect of estrogens on plasma lipid levels (274). This represents one of few attempts to account for the sex differential in human atherogenesis. Its validation would appear to demand the demonstration of significant sex differences

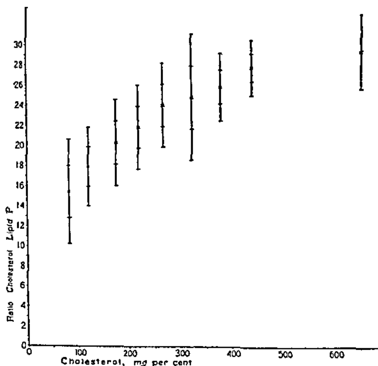


Fig. 7. The relation of cholesterol to the serum $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio—Peters, J. P. and Van Slyke, D. D (54), and Peters, J. P. and Man, E. B. (194)

TABLE XX

Plasma $\frac{\text{Total Cholesterol}}{\text{Lipid Phosphorus}}$ (C/P) Ratios in Patients with Various Clinical States—Gertler, M. M. (656)

Clinical Condition	Plasma Total Cholesterol mg %	Plasma Lipid Phosphorus mg %	C/P Ratio
Normal Female	245	12.2	20.1
Normal Male	225	11.3	19 0
Normal Mesomorph	223	12.0	19 0
Mesomorph with Coronary Heart Disease	294	13.2	23 0
Normal Ectomorph	195	11.0	18.0
Ectomorph with Coronary Heart Disease	250	11.5	21.7
Hypothyroidism	380	13.6	28.0
Hyperthyroidism	156	7.5	20.7
Nephrosis	493	15.4	32 0

Xanthomatosis	1045	52.0	20.1
Hyperlipemia	450	14.0	32.0
Biliary Cirrhosis	1023 530	74.0 38.0	13.8 14.0
Hypertension	210	11.0	19.1
Orchiectomy Pre-operative	208	9.6	21.5
Post operative	225	9.8	23.0
Diabetes Mellitus	385	14.3	26.9

in plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratios. However, as is apparent from Tables XVII, XVIII and XX, no such differences are demonstrable.

Further clinical studies on the significance of plasma C/P ratios have been accomplished by Gertler, *et al.* (107–111). These workers found that C/P ratios were significantly elevated in groups of patients with coronary atherosclerosis (*cf.* Table XV). They concluded, "The difference in total cholesterol: phospholipids ratio between the coronary disease group and the two healthy groups indicates a disturbance in lipid interrelationships in coronary heart disease. This is suggestive of a disturbance in serum colloidal make-up in the coronary disease group" (108).

Since their coronary disease group exhibited an increase in both mean plasma C/P ratio and total cholesterol concentration (Table XV), it is difficult to assay which change was decisive for atherogenesis. Similarly, the validity of Ahrens and Kunkel's conclusions concerning the significance of the C/P ratio depends on demonstrating the absence of intensified atherogenesis in patients with biliary obstruction, hypercholesterolemia and normal C/P ratios.* A larger series of cases is essential to verify this phenomenon

Recently, Gertler compiled data on plasma C/P ratios in patients with various clinical states (Table XX). Patently, several disease entities in which intensified atherogenesis is common exhibit elevated C/P ratios. Contrariwise, several in which atherogenesis is minimal exhibit normal or low C/P ratios However, inconsistencies are apparent, e.g., males as compared with females, xanthomatosis cases, hy-

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[illegible] that they not infrequently ex- [illegible] ratios [illegible] olemic [illegible] to that [illegible]

TABLE XXI

Comparison of Plasma C/P Ratios in Patients with Coronary Heart Disease and in *Normal Persons Having* Similar Plasma Total Cholesterol Levels—From Gertler, *M. M. et al.* (107, 656), and Peters, J. P. and Van *Slyke, D. D. (54)*

	Plasma Total Cholesterol of Gertler's Patients mg %	Plasma C/P Ratios of Gertler's Patients	Normal Plasma C/P Ratios for Given Level of Plasma Total Cholesterol—from Peters and Van Slyke
Control Group	224	19	22
Coronary Disease Group	287	23	25
Control Group—Mesomorphs	223	19	22
Coronary Disease Group—Mesomorphs	294	23	25
Control Group—Ectomorphs	195	18	21
Coronary Disease Group—Ectomorphs	250	22	23

pertensives, eunuchs. In these, the correlation between level of C/P ratios and susceptibility to atherosclerosis is poor.

A further point is in order here: Gertler and associates imply that the higher C/P ratios in their coronary disease vs. their control group represent abnormal elevations. However, the coronary group has higher plasma cholesterol levels (Tables XV and XX). According to Peters and Van Slyke (54), C/P ratios are normally higher with higher plasma cholesterol levels. It would therefore appear necessary to compare C/P ratios in a normal vs an atherosclerotic group *equated with respect to plasma cholesterol levels.* Higher C/P ratios in the latter group would be a far more significant finding. The importance of this point is emphasized by a comparison of data in Tables XV, XX and Figure 7. All the C/P ratios exhibited by the several coronary disease groups fall below values cited by Peters and Van Slyke as normal for the given plasma cholesterol level (Table XXI). Hence it has not been definitively demonstrated that the C/P ratio is abnormally elevated in the coronary disease groups.

Recently, Gofman presented data clarifying factors involved in determining C/P ratios in human plasma (153, 567). "A relative comparison of S_f ratios with cholesterol-phospholipid ratios indicates that molecules from S_f 3 to 100 have weight ratios of cholesterol to phospholipid in the neighborhood of one. Molecules of S_f 10–20 classes on the basis of our most recent determinations may be as high as 1.3. However, the high-density lipoproteins, probably corresponding to the alphalipoproteins that Dr. Barr mentioned, have a weight ratio of cholesterol to phospholipid in the neighborhood of one half. The overall serum cholesterol-phospholipid ratio is therefore determined more by the relative amount of high-density lipoproteins compared to low-density lipoproteins than it is by the distribution of cholesterol among the group from 3 to 100 S_f" (567) (cf. pages 99, et seq. and 114, *et seq.*).

Clearly, additional clinical research is indicated to evaluate further the relationship of C/P ratios to atherogenesis in man.

CHOLESTEROL DERIVATIVES

Other workers have taken a different approach to the problem of the plasma state of aggregation of cholesterol and its relation to atherogenesis. These investigators postulate that cholesterol derivatives may be the key atherogenic stimuli. They suggest that these may appear and operate pathogenically in the presence of normal plasma total cholesterol concentrations.* Along these lines, Kendall and associates investigated the atherogenic potential of several oxidation products of cholesterol isolated by Ruzicka from lipid extracts of atherosclerotic human aortas (56). They reported the identification of small amounts of one such substance (7-ketocholesterol) in sera of dogs with experimental atherosclerosis (280). They also studied the effects of giving rabbits single intravenous injections of oxidized colloidal cholesterol solutions containing 7-ketocholesterol and 7-hydroxycholesterol. They observed intimal deposition of lipid within 24 hours (56, 281, 282). Repeated intravenous injections of such colloidal cholesterol solutions induced sustained hypercholesterolemia and atheromatous lesions (cf. page 77). However, at least 20% unoxidized cholesterol was present in their aerated solutions (283). Hence it is not possible to ascertain whether the endothelial cells selectively imbibed the oxidized or unoxidized compounds. Neither Altschul (284) nor Cox and Spencer (285) were able to in-

*The preferred Schonheimer Sperry method of analysis for plasma cholesterol (278), involving acetone-alcohol extraction of plasma, precipitation

duce vascular lesions in rabbits by feeding or injecting 7-ketocholesterol. Further, Altschul obtained indifferent results in rabbit experiments with several other derivatives of cholesterol (284). Obviously this work on the role of oxidation and reduction products of cholesterol in atherogenesis is in its earliest stages. Definitive information has yet to be obtained. From present knowledge, it would appear that cholesterol itself, not its derivatives, is the offending atherogenic stimulus.

BOUND AND UNBOUND CHOLESTEROL

Forbes, *et al.*, recently developed a new method for studying the state of aggregation of cholesterol in plasma. These workers fractioned serum cholesterol chemically into bound and unbound components, the latter presumably being detached from protein (286). They found that a small fraction of normal plasma cholesterol is readily extractable with chloroform at 5°C from frozen, dried plasma. They further showed that this readily extractable fraction is slightly elevated in hypothyroid and some diabetic patients. It is markedly increased in nephrotic humans and hypercholesterolemic rabbits. Duff and Payne utilized this technique in an experimental study of cholesterol-induced atherosclerosis in rabbits. These workers were unable to demonstrate a correlation between atherogenesis and concentrations of unbound cholesterol and phospholipid in their cholesterol-fed animals (287). In view of the technical simplicity of the extraction procedure, this approach merits further study despite Duff and Payne's negative results. Electrophoretic and ultracentrifuge studies would be of value to delineate the precise state in plasma of so-called "unbound" cholesterol. From extant data, it would appear that all the plasma lipids exist naturally as constituents of complex lipoprotein molecules of high molecular weight (556).

CLASSES OF CHOLESTEROL-BEARING LIPOPROTEIN MOLECULES (ULTRACENTRIFUGE STUDIES)

Of the recent studies on the state of aggregation of cholesterol in plasma, probably the most significant are those of Gofman, *et al* (152, 153). As already indicated, these workers developed an ultracentrifuge method (289) for the identification and quantitative characterization of individual lipid-bearing protein molecules of plasma (288). They used these quantitative ultracentrifuge techniques to clarify whether in atherosclerosis "the physico-chemical nature of the blood lipids might be of more significance than the analytic lipid levels themselves" (152).

"In the work with atherosclerosis our concern is primarily with lipid and lipoprotein molecules of densities close to 1.00 Gm./cc. By adjusting the density of the solution being studied with sodium chloride to a value of 1.063, the solution is thereby made more dense than the molecules themselves, with the result that all molecules of lesser density undergo flotation. In fact in this work we preliminarily float all the lipids and lipoproteins of a class less dense than 1.063 to the surface in a preparative ultracentrifuge, pipet off the top fraction containing them, and then study the group for its individual constituents by the analytic ultracentrifugal method. . . . The customary unit of migration is the Svedberg, named in honor of T. Svedberg who invented the ultracentrifuge and developed the theory of its use. A molecule which undergoes sedimentation at a rate of 5×10^{-13} cm per sec. per unit field of force is said to have an S value of 5, or to be a molecule of the S 5 class. We have adapted this term for flotation runs such that a molecule which undergoes flotation at a rate of 5×10^{-13} cm. per sec. per unit field of force has an S_f value of 5, or is a molecule of the S_f 5 class.

"The group of lipoproteins and lipids in human serum of densities less than 1.063 Gm./cc. may consist of as many as ten species of molecules, the number being variable from individual to individual. Several of these molecules are cholesterol-bearing molecules, and in fact generally carry a large fraction of the total serum cholesterol. These components are readily characterized by their flotation rates under specified conditions or by their hydrated densities. For present purposes the flotation rates are most useful

"The various classes of molecules found in serum by these studies are the following·

"(a) Species which migrate with S_f values greater than 75 S_f units. These include the well-known chylomicrons and aggregates of much smaller dimensions than the chylomicrons. The concentration of species in this entire class is increased following fat-containing meals and hence represents part of the alimentary lipemia. We have found no correlations between components of S_f greater than 75 units and the presence of atherosclerosis in humans.

"(b) Species which migrate with S_f values between 30–70 S_f units. These species constitute the major fraction of alimentary lipemia and are greatly modified in concentration with relationship to meals. Certain of these components are cholesterol-bearing molecules and their relationship to atherosclerosis is under investigation.

"(c) Species of discrete classes which migrate with rates between 10 and 20 S_f units. These components, whose molecular weights are in the neighborhood of 3,000,000,* appear definitely related to the presence of atherosclerosis in

*The molecular weight of cholesterol is 404 65; the molecular weight of the oleic acid ester of cholesterol is 600 10 Since the molecular weight of a

$\frac{.30 \times 3,000,000}{404\ 65}$ or more than 2000 cholesterol molecules! (L N.K & J.S).

humans. There are occasionally present in addition molecules of the S_f 20–30 class, some of which appear to have significance like that which characterizes the molecules of the S_f 10–20 class, but the role of the S_f 20–30 components requires further clarification. The S_f 10–20 class contains at least three separate species of molecules, each approximately 30% cholesterol by weight, which at present appear to be of *equivalent significance with respect to atherosclerosis.*

"(d) Species which migrate with S_f rates between 3–8 S_f units. These molecules are important cholesterol, phospholipid, and protein-containing substances, loosely referred to in the general literature as the β_1-lipoprotein. Actually this component may exist as a single component or as a multiple group of components in an individual case. This component or set of components, carrying a major fraction of the serum cholesterol, is present in every one of some 4000 samples studied, at concentrations varying from individual to individual, but at an essentially constant level for a given individual from time to time. This set of components does not of itself appear to be related to atherosclerosis" (152).

As is evident from Table XXII, ". . . The actual lipid constituents which are transported via the different lipoproteins are quantitatively and qualitatively different. The major neutral fat (glyceryl ester) bearing molecules are those of S_f 17 and higher whereas in general the major cholesterol-bearing molecules are those of S_f 17 and lower. Thus since enormous variations in the relative concentra*tions of the individual lipoproteins exist from one person to* another, it should be evident that a determination of the total lipid level by the usual classic chemical technics will fall far short of an adequate description of the true manner in which the lipids are being transported" (556).

In more recent studies, Jones, Gofman, *et al.*, obtained evidence indicating that these various circulating lipoprotein

TABLE XXII

Chemical Composition of Human Plasma Lipoproteins of Various S_f Classes—Jones, H. B. *et al.* (556)

	S_f 4	S_f 6	S_f 8	S_f 10	S_f 13	S_f 17	S_f 17–40	S_f 40–40,000
Total cholesterol	~ 30%	Decreasing steadily →						5%
Fraction of cholesterol esterified	~ 75%	Decreasing steadily →						0%
Phospholipid	~ 25%	Decreasing steadily →						~ 5%
Protein	~ 25%	Decreasing steadily →						5%
Glyceryl ester	← Absent or very low % in this range →						Increasing steadily →	75–85%

molecules are in fact transport vehicles for lipids in plasma (556). ". . . All the molecules from S_f 40,000 down to S_f 4 (and possibly into the high density class) represent a sequence of molecules in a metabolic chain involved in the ultimate utilization of glyceryl esters and/or fatty acids" (556). Jones, et al, summarized the available evidence indicating that components of high S_f value are progressively transformed into those of the lower S_f classes. They found that the average lifetime of all the lipoprotein species of the low density group is of the order of several hours. Based on these tracer and other studies, they arrived at the further conclusion that the normal pattern is one showing no appreciable concentration of any lipoproteins above a certain limit (S_f 6) for the human. "Deviating from this simple pattern there is a regular sequence of types of patterns which may be regarded by itself as a progression of a lipid metabolic defect reflecting itself in the lipid transport mechanism" (556). The nature of this progression is illustrated in Table XXIII from Jones, et al (556). Kendall's studies led him to a similar interpretation of the significance of plasma S_f patterns (566).

Having identified various groups of lipoprotein molecules in plasma, Gofman, et al, proceeded further to determine their relationship—if any—to atherosclerosis. Their summary best presents the essence of their findings:

"The presence of a class of lipid and lipoprotein molecules in the serum of man and the cholesterol fed rabbit, associated with atherosclerosis in both species, has been demonstrated.

"These molecules do not represent any part of the acute alimentary lipemia.* The presence of these cholesterol-bearing lipid and lipoprotein molecules cannot be predicted

*This demonstration of a lack of correlation between lipoproteins involved in atherogenesis and alimentary hyperlipemia throws additional doubt on the validity of Moreton's concepts (cf page 86) (L.N.K. & J.S.).

TABLE XXIII

Lipoprotein Transport as a Measure of Lipid Metabolic Defect (Human)—Jones, H. B. *et al.* (556)

"Normal" Pattern

(1) Lipoproteins of S_f 4 and/or S_f 6 present at low or moderate concentrations Minimal levels of higher S_f components except for transient elevations in S_f 30–40,000 following fatty meals.

"Minimal" Defect

(2) Lipoproteins of S_f 4 and/or S_f 6 at increased concentrations but without any increase in higher S_f components as compared with (1).

"Minor" Defect

(3) Lipoproteins of S_f 4 and/or S_f 6 plus S_f 8 in increasing concentration

Progressively "More Severe" Defect

(4) S_f 4 + S_f 6 + S_f 8 + S_f 10
(5) S_f 4 + S_f 6 + S_f 8 + S_f 10 + S_f 13
(6) S_f 4 + S_f 6 + S_f 8 + S_f 10 + S_f 13 + S_f 17
(7) S_f 4 + S_f 6 + S_f 8 + S_f 10 + S_f 13 + S_f 17 + S_f 17–20
(8) S_f 4 + S_f 6 + S_f 8 + S_f 10 + S_f 13 + S_f 17 + S_f 17–20 + S_f 20–40
(9) S_f 4 + S_f 6 + S_f 8 + S_f 10 + S_f 13 + S_f 17 + S_f 17–20 + S_f 20–40 + S_f 40–40,000
(In this group the S_f 40–40,000 can be of transient existence following meals or may be sustained even post absorptively.)
(10) "Most Severe" Defect
As in (9) except that the S_f 4 and S_f 6 may be depressed to quite low concentrations. (This may be regarded as a general shift toward higher S_f lipoproteins, and is comparable to that which appears in the rabbits in the later phases of cholesterol-Wesson oil feeding.)

from the analytic total serum cholesterol level Partial dietary restriction of fat and cholesterol in man results in a gradual decrease in the serum level of such molecules over a period of weeks to months.

"These molecules are present with a much higher frequency and at higher concentrations in patients who have survived a myocardial infarction than in corresponding individuals without known vascular disease. The presence of

these molecules with increased frequency in other diseases associated with excessive atherosclerosis (diabetes mellitus, the nephrotic syndrome, hypothyroidism, hypertension and coronary insufficiency) supports the hypothesis of their association with atherosclerosis" (Table XXIV) (152).

These conclusions of Gofman, Lyon and co-workers have recently been subjected to serious criticism by Keys (540). He questioned whether the data presented by Gofman, *et al.*, actually support their fundamental thesis. This thesis, it will be recalled, is as follows: "The basic premise of this research was that there might be a defect in the molecules which transport fats and cholesterol that could be more intimately related to the pathogenesis of atherosclerosis than are the total analytical levels of the various lipids themselves, e g., serum cholesterol levels" (539).

Keys noted that Gofman, *et al*, did not submit their raw data to statistical analyses, correlating the actual relationships among total cholesterol levels, S_f 10–20 levels and atherosclerosis. He therefore proceeded to carry out appropriate analyses of the data presented by Gofman, *et al.*, in their two initial publications (152, 153). His findings led Keys to these conclusions:

"From an analysis of published data it appears that there is an important correlation between the concentrations in the blood serum of total cholesterol and of the 'giant' molecules or aggregates (G) characterized by -10 to -20 Svedberg units in the ultracentrifuge.* The correlation coefficient in both normal men and in men with myocardial infarctions is of the order of at least 0 5 to 0.6.

"As a criterion to discriminate between clinically normal and men with the important degree of atherosclerosis rep-

*This is a very different conclusion from that of Gofman, et al "The presence of these cholesterol-bearing lipid and lipoprotein molecules cannot be predicted from the analytic total serum cholesterol level" (152) (L N K and J.S).

TABLE XXIV

Mean Plasma Concentrations of Cholesterol-bearing Lipoprotein Molecules of the S_f 10–20 Class in Various Disease States—Gofman, J. W. *et al.* (152)

	Mean Plasma Concentration of S_f 10-20 Molecules, mg %				
	Normals	Coronary *Atherosclerosis**	Hypothyroids	Diabetics	Hypertensives
Males, Age 20–40 yrs.	15	—	31	19	30
Males, Age 40–70 yrs	16	33	55	18	25
Females, Age 20–40 yrs.	7	—	—	22	—
Females, Age 40–70 yrs.	13	49	27	21	25

*Really myocardial infarction and coronary insufficiency

resented by myocardial infarction, the measurement of G is not superior to and may well be inferior to the measurement of total cholesterol in the serum.

"Extremely low values of G in serum are rare in men with infarcts, hence the finding of such low values may be useful in estimating the likelihood of normality. On the other hand, very high values of total cholesterol in the serum tend to be more frequently related to atherosclerosis than are correspondingly high values of G.

"There is no evidence that combining G and total cholesterol criteria will improve predictions of abnormality made from cholesterol measurements alone.

"Data from cholesterol-feeding experiments with rabbits show the severity of atherosclerosis which results is highly correlated to about the same degree with both G and total cholesterol levels in the serum, but that the effective serum levels are beyond the range usually seen in man.

"At the present time, it is entirely unjustified to attribute to G measurements any special virtue beyond that for simple cholesterol measurements for the prediction of atherosclerosis or the estimation of the activity of the atherosclerotic process" (540).

It is to be noted that these conclusions of Keys were derived from analyses of data presented by Gofman, *et al.*, in their first three publications (152–153). Their additional findings (538, 539, 556, 567) would appear to compel a modification of the criticisms Keys leveled. Some of these data on males with myocardial infarction vs. normals are presented in Figure 8 and Table XXV (556). Analysis of these data led to the following conclusions: "Thus for both age groups [41–50, 51–60 years of age] there is a segregation of infarcts from normals by S_f 12–20 measurements, independent of the serum cholesterol. However, while the 41–50 year age group is segregated approximately as well by serum cholesterol measurement, independent of S_f 12–20, the 51–60

TABLE XXIV

Mean Plasma Concentrations of Cholesterol-bearing Lipoprotein Molecules of the S_f 10–20 Class in Various Disease States—Gofman, J. W. *et al.* (152)

	Mean Plasma Concentration of S_f 10-20 Molecules, mg %				
	Normals	Coronary Atherosclerosis*	Hypothyroids	Diabetics	Hypertensives
Males, Age 20–40 yrs.	15	—	31	19	30
Males, Age 40–70 yrs.	16	33	55	18	25
Females, Age 20–40 yrs	7	—	—	22	—
Females, Age 40–70 yrs.	13	49	27	21	25

*Really myocardial infarction and coronary insufficiency.

year age group infarcts are not significantly segregated by serum cholesterol measurements" (556).

Pooling the two age groups, and comparing 156 cases of myocardial infarction with 124 normals age 41–60 years, Jones, et al., obtained the data summarized in Table XXVI (556). They concluded: ". . . The S_f 12–20 measurement significantly segregates myocardial infarcts from normals, independent of the serum cholesterol levels. However, for the same group, serum cholesterol does not significantly segregate myocardial infarctions from normals, independently of the S_f 12–20 levels" (556).

Expressed somewhat differently, these data demonstrate the following:

". . . At any range of serum cholesterol, even including hypercholesterolemia, the patients with atherosclerosis or diseases predisposing to atherosclerosis show higher S_f 10–20 levels than do presumably normal individuals. For example, at a low cholesterol such as 200–225 mg.%, patients with coronary disease show higher S_f 10–20 levels than do normals. Likewise, in the hypercholesterolemic range, say 300–350 mg.%, patients with coronary disease show higher S_f 10–20 levels than do normals who carry the same total cholesterol" (539).

Further:

"Hypertensive patients who have coronary artery disease (presumably atherosclerotic in origin) show higher S_f 10–

---

the mean S_f 12–20 level can be read for [illegible]

mean S_f 12–20 level at any cholesterol level in normals and myocardial infarcts, respectively. Lines C and D show the mean choles- [illegible]

[illegible] and 92 myocardial infarctions)—Jones, H. B., et al (556).

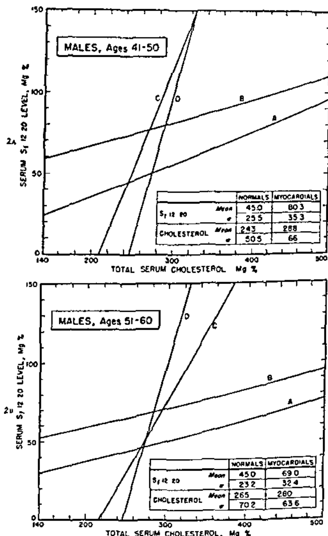


FIG. 8. The regression lines for normals and myocardial infarctions for cholesterol values and S_f 12–20 values from a study of both determinations on the same serum sample from each individual considered. These lines are calculated from the correlation coeffi- [illegible]

II. Matched by S_f 12–20 Levels

(a) 41–50 yr. age group

	No of Cases	Mean Serum Cholesterol	Mean S_f 12–20 Level
Myocardial infarcts	55	292.3 mg %	74 3 mg %
Normals	55	273.2 mg %	74.3 mg %

Difference in cholesterol = 19.1 mg % ± 6.4
(Significant $p < 1\%$)

(b) 51–60 yr. age group

	No of Cases	Mean Serum Cholesterol	Mean S_f 12–20 Level
Myocardial infarcts	69	283.3 mg %	60.4 mg %
Normals	69	276 3 mg %	60.4 mg %

Difference in cholesterol = 7 0 mg % ± 8.1
(not significant)

*Lipoprotein and cholesterol determinations were done on aliquots of the same serum sample from each individual in the study.
**The error estimated is the standard error of the difference of the means appropriately calculated for a matched series, with the correction for intercorrelation.

TABLE XXV

Myocardial Infarcts Matched with Normals by Serum Cholesterol Levels or by S_f 12–20 Lipoproteins*—Jones, H. B. *et al.* (556)

I. Matched by Serum Cholesterol

(a) 41–50 yr age group

	No of Cases	Mean Serum Cholesterol	Mean S_f 12–20 Level
Myocardial infarcts	55	291.3 mg %	73.8 mg %
Normals	55	291.5 mg %	53.7 mg %

Difference in S_f 12–20 = 20.1 ± 5.5**
(Significant $p < 1\%$)

(b) 51–60 yr. age group

	No of Cases	Mean Serum Cholesterol	Mean S_f 12–20 Level
Myocardial infarcts	69	276 mg %	62.4 mg %
Normals	69	276 mg %	49.0 mg %

Difference in S_f 12–20 = 13.4 ± 3.5
(Significant $p < 1\%$)

in its early stages. The fundamental significance of its findings cannot yet be definitively evaluated. If final judgment is for the present withheld, however, this in no way detracts from the importance of the new research path being explored by Gofman, Lyon and co-workers. Their approach and the findings stemming from it point the way to a possible resolution of a number of unclarities plaguing investigators in the atherosclerosis field. Normocholesterolemia, diet, alimentary lipemia, age—the relationship of each of these and other factors to atherosclerosis will almost certainly be clarified by ultracentrifuge studies of classes of cholesterol-bearing lipoprotein molecules. In any case, the results of this work to date—regardless of possible areas of disagreement concerning their interpretation—are major new sources of evidence in support of the cholesterol concept of atherogenesis.

CHEMICAL AND ELECTROPHORETIC MICROFRACTIONATION OF PLASMA PROTEINS AND LIPOPROTEINS

Finally, note must be taken of the fact that ultracentrifuge analysis is not the only avenue of approach to the problem of the role of lipoproteins in atherogenesis. Other techniques, e g, biochemical (63) and electrophoretic (236, 289, 290) are available for such studies. To date they have been utilized to only a limited degree. Thus a preliminary report by Gofman indicated that alterations in α- and β-globulins occur in conjunction with emergence of lipoproteins of the higher S_f classes (153). Further, Pearsall and Chanutin correlated plasma lipid levels and protein fractions in humans with various diseases However, the possible relationship of their findings to atherogenesis was not analyzed (236). Another recent report along similar lines indicated that cholesterol-fed rabbits developed β- hyperglobulinemia

20 levels than do individuals with uncomplicated hypertension.

"Diabetic patients with vascular disease (hypertension and/or coronary artery disease) show higher levels than do diabetic individuals who do not show these complications" (538).

". . . Both the average blood level of S_f 10–20 molecules as well as the frequency of occurrence of high levels (over 60 mg. %) are significantly (p = 0 03) greater for patients experiencing recurrent [myocardial] infarction than for those who did not have a recurrence.

". . . Moderate reductions [in S_f 10–20 levels] produced by diet* are capable of giving these individuals [with known coronary artery disease] significant protection from a high recurrence rate of myocardial infarction. All these data are consistent with the hypothesis that the reduction in S_f 10–20 lipoprotein level may have lessened the progression of atherosclerotic disease" (539).

Obviously this departure in atherosclerosis research is still

TABLE XXVI

All Myocardial Infarctions from 41–60 Years Matched by Age and by S_f 12–20 or Cholesterol from the Regression Equations —Jones, H. B. *et al.* (556)

(A) Matched at the same cholesterol levels·		
	(for 156 cases)	S_f 12–20 levels average 23.1 mg % higher in myocardial infarcts than in normals.
(B) Matched at the same S_f 12–20 levels		
	(for 156 cases)	Serum cholesterol averages 1.1 mg % less in myocardial infarcts than in normals.

*In contrast to the depressing effect on S_f 10–20 levels of a conventional low fat low cholesterol diet, Hatch and Kendall reported an increase or no change with the rice diet in hypertensive patients (cf. pages 37, 42) (658) (L.N.K. and J.S.).

patterns were found (558). "These include a tendency to reduction of albumin and alpha lipoprotein and a relative and absolute increase in beta lipoprotein . . . These changes may be apparent without hypercholesterolemia or recognizably significant elevation of the cholesterol-phospholipid ratio of the unfractionated plasma . . . In many diabetics they are apparent before any vascular complications of the disease are clinically recognizable. They were seen in two cases of familial xanthoma tendinosum. They are present to an extreme degree in patients with the nephrotic syndrome. It is suggested that future attempts to relate lipids of the plasma to the deposition of lipids in tissue must take account of their combinations with protein; also, that further exploration of protein-lipid relationships may be rewarding

TABLE XXVII

Microfractionation of Plasma Proteins Accomplished by Method 10 of Cohn, E. J. *et al* (550)—Barr, D. P. (541)

Fraction A(IV, V, VI)	
	Albumin
	Alpha$_1$ lipoprotein
	Alpha$_2$ glycoprotein
	Beta$_1$ metal combining protein
Fraction B(II)	
	Gamma globulins
Fraction C(III$_0$)	
	Beta$_1$ lipoprotein
	Beta$_1$ lipid poor euglobulin
	Ceruloplasmin
Fraction D(I, III$_{1,2,3}$)	
	Fibrinogen
	Prothrombin
	Cold insoluble globulin
	Plasminogen
	Isoagglutinins

in conjunction with hypercholesterolemia (291). Finally, Barr recently reported on abnormal variations in the distribution of cholesterol-bearing lipoproteins in the plasma of individuals with atherosclerosis (541). For his analytical procedure, he utilized a new modification (Method No. 10 of Cohn, *et al.*) for biochemical microfractionation of plasma proteins (63, 550). The separation of plasma proteins effected by this method is indicated in Table XXVII and Figure 9 (541, 557). For purposes of atherosclerosis research, the significant feature is the separation of the plasma lipoproteins into two distinct fractions. α_1- and β_1-lipoproteins (Fig. 9) are segregated in Fractions A and C respectively (Table XXVII).* Chemical analysis of the fractions revealed the following facts: Practically all of the plasma cholesterol is contained in Fractions A and C (α_1- and β_1-lipoproteins), approximately 30% in the former and 70% in the latter (Table XXVIII—note sex difference) (557). Fractions A and C contain similar amounts of lipid phosphorus; hence the $\frac{\text{cholesterol}}{\text{lipid Phosphorus}}$ ratio is low in Fraction A and high in Fraction C (Table XXIX) (557).

In persons with coronary atherosclerosis, or with disease predisposing to atherosclerosis (diabetes, nephrosis, xanthomatosis), significant departures from the foregoing normal

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*Neither Fraction A nor C is a distinct entity, rather, each contains between 3 and n molecules (556, 567). The α_1-lipoproteins are high density molecules (densities of 1 07–1.12). These are not flotated in the usual ultracentrifuge runs of Gofman, *et al*, wherein the density of serum is raised to [illegible] serum is raised to 1 24 with a [illegible] roteins are flotated ultracen- [illegible] β_1 lipoproteins are low density [illegible] of density 1 063 at the fol- [illegible] (densities of 1 050–1 035); [illegible] abnormal patterns—S_f 2–6 plus S_f 8–17 (densities of 1.029–0 99) and S_f 17–40,000 (densities of 0 99 and <) Since molecules of classes S_f 10 and above emerge in plasmas of cholesterol fed rabbits, and these are all β_1-lipoproteins, the finding of β-hyperglobulinemia in conjunction with hypercholesterolemia (291) is not unanticipated.

both in clarifying the pathogenesis of atherosclerosis and in aiding in its early recognition" (558).

The authors noted that the data presented did not definitively establish a relationship between the changes observed and atherogenesis. The possibility exists that these alterations are non-specific in origin. Nevertheless, they correctly stressed that future efforts to relate the lipids of plasma to lipid deposition in tissues, particularly arteries, must take into account their combinations with protein, and particularly with α- and β-lipoprotein (541). Certainly further work along these lines is clearly indicated.

In concluding this discussion on the colloidal state of aggregation of cholesterol in plasma, we wish to emphasize that this relatively new approach to the atherosclerosis problem has already borne considerable fruit. This estimate in no way detracts from the fact that the final significance of certain suggestive correlations, e g., between the C/P ratio and atherosclerosis, between S_f 10–20 lipoproteins and

TABLE XXIX

Lipid Phosphorus and $\frac{\text{Cholesterol}}{\text{Lipid P}}$ (C/P) Ratios in Plasma and Plasma Fractions of Presumably Normal Persons*—Russ, E. M. *et al.* (557)

	Lipid P** mg %	C/P
Whole plasma	10 1	23.8
Fraction A (a_1)	4 3	12.8
Fraction C (β_1)	4 6	33 8

*38 cases, 17 males (2 age 25, 15 age 45–63); 21 females (11 age 22–34, 10 age 51–62 yrs)
**phospholipid/25

TABLE XXVIII

Distribution of Cholesterol in Plasma Fractions of Presumably Normal Persons—Russ, E. M. *et al.* (557)

Sex	Age Yrs.	No. of Cases	Whole Plasma Cholesterol mg %	Cholesterol in Fraction A (a_1) %	Cholesterol in Fraction C (β_1) %
Male	18–35	24	197	25.2	69.3
Female	18–35	20	187	34.3	58.4
Male	45–65	21	239	22.9	71.2
Female	45–65	20	252	23.4	71.5

simetry, chromatography and immunochemical analysis will be applied for the further "dissection" of the plasma cholesterol-containing lipoprotein colloidal micelle. Can an antibody titer be built up against lipoproteins of various S_f classes? Can such lipoproteins be "neutralized" immunologically? Will atherogenesis be affected thereby? Perhaps of greater significance will be the application of tracer methods to the study of factors regulating lipid and protein synthesis, turnover and release into the plasma. Why under certain conditions of diet or disease do new classes of cholesterol-bearing lipoproteins emerge? These and other questions related to cholesterol metabolism and atherosclerosis can best be answered by tracer methods It need only be re-emphasized, in launching and organizing this multifaceted new attack on metabolic-biochemical factors in atherosclerosis, that its goal is to deepen our understanding of the relationship of cholesterol to atherosclerosis. Present knowledge fully supports the concept that this is the key aspect of the atherosclerosis problem. Moreover, in applying these methods of physical chemistry in this field, attention must remain focussed on the problem at hand: Enhancing our understanding of a pathobiological process, atherogenesis, for the ultimate purpose of curbing and eliminating it. The pathologic lesion remains our primary concern!

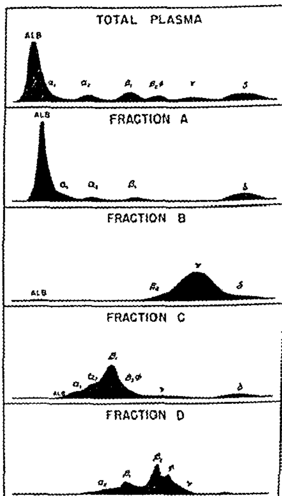


Figure 9 Electrophoretic patterns (from Ascending Limb) of normal plasma and its four fractions—Russ, E M., *et al.* (557).

atherosclerosis, between other classes of lipoprotein molecules and atherosclerosis, remains to be definitively ascertained.

It may also be anticipated that such techniques as electron microscopy, x-ray diffraction analysis, nephelometry, visco-

A thorough review of the work in this field since 1911 is beyond the scope of this monograph. Actually the need for such an effort is obviated by the several extant monographs and critical reviews, of early and recent vintage, to which the reader is referred (3, 4, 7–11, 28–31, 38, 53, 55–57, 80, 86, 102, 186, 267–269, 284, 294–297, 551–565).

Our purpose here is rather to summarize the 10 years' or more experience of this department on experimental atherosclerosis, principally in the chick. In the course of this presentation frequent reference will be made to previous and contemporary studies in other species, as they relate to our findings.

We have purposively elected to present our work in the form of a series of questions and answers. The latter, based on experimental findings, are necessarily partial, incomplete and often tentative. This form of presentation is utilized to highlight past and present problems in the field, and to aid in illuminating the road ahead. In taking this approach, we aim to stress the fact that this monograph is a progress report—a survey and summing up of where we stand today and where we have to go tomorrow. Throughout we have consciously applied restraint in drawing broad inferences from existent experimental data to human atherosclerosis, although—as already indicated—the interplay between clinical and laboratory research is a key to progress in this field We leave to the reader the prerogative to make whatever such deductions he wishes.

WHY THE CHICK AS EXPERIMENTAL ANIMAL?

In the original probe experiments on atherosclerosis undertaken in this department over a decade ago by Katz, Friedberg, Sanders, Hurwitz, Megibow and Carlen, rabbits were used as experimental animals. They proved to be far from satisfactory. Much of the initial data collected was in-

IV EXPERIMENTAL ATHEROSCLEROSIS

UNDOUBTEDLY STUDIES OF HUMAN MATERIAL HAVE IN THE past contributed considerably to the knowledge of atherosclerosis. They will continue to make a contribution in the future. However, the very chronicity of the atherosclerotic lesion, together with the limitations placed on investigative research in living man, highlights the extent of our reliance on animal experimentation for a solution of this problem. In this, as in other fields, proper experiments on animals are as vital for human welfare as the sacrifice of animals for food. Man's present and future progress is as dependent on the experimental approach as in the past. Science must continue to adhere to the tried method of initial careful testing of new procedures on species other than man. Only when such trials appear fruitful, is it justified to extend them cautiously to the species which concerns us most, *homo sapiens*.

Prior to the pioneer work with cholesterol three decades ago (16–27), numerous experimental attempts to reproduce the lesion of human atherosclerosis in animals had uniformly met with failure (cf. page 5 and references 4 and 38). Except for avian estrogen-induced atherosclerosis (a lesion secondary to endogenous, endocrine-stimulated hypercholesteremic hyperlipemia) (292, 293), experimental atherosclerosis has to this day been successfully produced only by cholesterol administration. In the positive sense, therefore, experimental atherosclerosis begins with Ignatowski and Anitschkow.

neous. Toward this end, Dauber and Katz placed two groups of 10-day-old cockerels on experimental and control diets. The latter received commercial chick starter mash of known composition. The experimental birds were given the same mash supplemented with 2.5–5.0% cholesterol plus approximately 20–25% cottonseed oil (298). All cholesterol-fed birds surviving beyond the 8th experimental week had gross atherosclerosis of the aorta and great vessels. Several had lesions in the coronary arteries which narrowed the lumina. The control cockerels were uniformly free of atheroma.

In a subsequent experiment designed to delimit the factors responsible for atherogenesis, it was demonstrated that cottonseed oil alone did not induce atheroma (see below). Likewise, simple underfeeding* did not result in atherogenesis (see below) Thus it was clearly established that the lesions resulted from cholesterol feeding (299).

In 1946 the horizon and methodology of experimental atherosclerosis in the chick were further broadened. Up to that time, two types of lesions were available for study, the spontaneous and the cholesterol-induced. In that year, Lindsay, *et al.* demonstrated that atherosclerosis occurred in cockerels having a prolonged endogenous hyperlipemia induced by implantation of estrogenic material (292, 293, 310). Horlick and Katz confirmed this finding and extended it by demonstrating that the lesions in stilbesterol-treated chicks were not of the spontaneous type (293). This signifi-

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*The experimental diets used in these early studies resulted in poor feed intake and stunted growth The chicks were grossly malnourished and nutritionally deficient Hence it was essential to rule out this factor in athero- [illegible]

[illegible]

feed intake, growth and development, even when placed on a diet of as much as 2% cholesterol beginning the first day of life (309) Therefore, while cholesterol is the offending agent inducing atherosclerosis in the chick, it is not nutritionally toxic, in the usual meaning of the word.

conclusive, and to this day remains in the department's unpublished files. When a more extensive program of research on experimental atherosclerosis was projected, it was deemed essential to proceed along lines obviating the serious criticisms leveled by many workers at the use of the rabbit for such investigations (4, 11, 54, 58, 186, 267). This species is herbivorous and does not normally ingest any cholesterol. Moreover, on its usual diet it may not naturally develop vascular lesions of the atherosclerotic variety. Finally experimentalists up to that time had experienced only limited success in consistently inducing gross atherosclerotic lesions by cholesterol feeding in species other than the rabbit. All these facts led many workers to question seriously the relevancy of rabbit cholesterol-induced atherosclerosis for an understanding of the pathogenesis of the human disease.

In seeking for a more suitable experimental animal, Dauber and Katz (298–300) selected the chick after a careful survey of the existing literature. Dauber advanced cogent reasons for making this choice in an attempt to reach beyond the then existent impasse: "Of all animals, birds have arteriosclerosis most closely resembling human arteriosclerosis. For this reason the experimental production of arteriosclerosis in birds was undertaken. . . . It was reasoned that it would be instructive to reproduce the disease in an animal which is naturally subject to the same type of vascular changes as man. The fact that the chick, like man, is omnivorous would give added significance to the results" (300).

Spontaneous avian arteriosclerosis had been extensively studied by Fox (301, 302). A few reports were in the literature concerning cholesterol-induced atherosclerosis in chicks (303–306). Since these experiments were carried out in small groups of birds of unknown or advanced age, with few controls, initial studies were essential to demonstrate that the lesions were experimentally induced and not sponta-

Hens develop nodular and ridgelike plaques of the descending thoracic and abdominal aorta essentially similar to the spontaneous lesions in roosters.

Microscopically the spontaneous atherosclerotic lesions of the abdominal aorta have a like morphology in both rooster and hen (300). Essentially the intima is focally thickened to a varying degree by fibrous tissue. The overlying endothelium is intact. The fibrous tissue comprising the intimal thickening is young and cellular in regions of little proliferation and also at the surfaces of large plaques. On the other hand, the deeper portions of thick plaques are composed of acellular collagenous tissue. Hyaline change in the collagen is not uncommon. In the depths of the plaques, close to the media, areas of mucoid degeneration may also be seen. In some of the lesions she studied, Dauber observed fusiform cholesterol slits and round calcific granules in these necrotic zones. On occasion she also noted large pale foam cells in the deeper layers of these fibrous plaques, as well as vasa vasorum and extravascular blood cells. Sudan IV stains of frozen sections sometimes revealed varying quantities of lipid in the depths of the thickened intima and also in the adjacent medial tissue. Often, however, no stainable lipid was demonstrated More recent pathologic studies by R. Pick indicate that almost without exception no lipid is seen in spontaneous lesions of birds reared in the laboratory throughout their life, consuming mash devoid of a cholesterol supplement (266). The sudanophilic material noted by Dauber in spontaneous lesions occurred extracellularly as globules and rodlike crystals in areas of degeneration, and intracellularly in the cytoplasm of fibroblasts adjacent to necrotic zones (300). Such lipid-bearing fibroblasts were also seen in areas of moderate intimal fibrosis which had not undergone degenerative changes Usually these cells lay in that portion of the thickened intima adjacent to the media, or in the middle of the plaque. We are currently in-

cant advance placed three lesions* at our disposal for study in the chick: the spontaneous; the cholesterol-induced, secondary to an exogenous source of dietary lipid; and the estrogen-induced, consequent upon an endogenous, endocrine-stimulated hyperlipemia. This unique versatility of the chick further enhances its utility in atherosclerosis research.

WHAT IS THE MORPHOLOGY OF ATHEROSCLEROSIS IN THE CHICK?

A. Spontaneous Atherosclerosis

Dauber gave a detailed description of the *morphology of spontaneous atherosclerosis* in domestic chicks reared commercially in the United States (300). Her studies included gross analysis of fresh and Sudan IV stained aortas, and microscopic examination of sections stained with iron-hematoxylin, orcein elastic tissue stain, Giemsa stain or fat stains. In the rooster, the abdominal aorta is by far the commonest site of change. Characteristically these spontaneous lesions are elevated, smooth, longitudinal white or yellow ridge-like thickenings of the abdominal aorta, extending from the *interrenal region to the bifurcation On rare occasions* small, pinhead-size, elevated, yellow plaques develop adjacent to the orifices of arteries branching from the aorta. These nodular elevations sometimes stain with Sudan IV, in contrast to the ridgelike thickenings, which tend to stand out by their failure grossly to take the fat stain. In no case have we seen gross atheroma of the foregoing types in the elastic aorta of roosters *(the ascending aorta and arch) either on simple* inspection or after staining with Sudan IV.

*From our point of view, a gross lesion with definite thickening of the arterial wall is the essential criterion for successful experimental production of atherosclerosis. We do not regard lipid infiltration *per se* of the arterial wall as atherosclerosis, although it may well be its precursor.

The pathogenesis in hens of this diffuse lipid infiltration of the elastic aorta has apparently been clarified recently by the experimental work in stilbesterol-treated birds. Cockerels with prolonged estrogen-induced hyperlipemia exhibit this same change. Probably, therefore, the spontaneous lipid infiltration of the elastic aorta in hens is a byproduct of the estrogen-stimulated physiologic hyperlipemia occurring during the egg-laying period. However, the pathogenesis of this finding in roosters remains obscure.

B. Cholesterol-induced Atherosclerosis

The *morphology of cholesterol-induced atherosclerosis* of the aorta and great vessels is also described in detail in the publications of Dauber and Katz (298, 299) and Horlick and Katz (311, 312). Our subsequent observations are essentially in agreement with these findings.

Horlick and Katz observed that gross cholesterol-induced lesions in both the thoracic and abdominal aorta may occur in birds after as little as 1½–2 weeks of experimental feeding (312). To our knowledge this represents the most rapid recorded induction of gross cholesterol-induced lesions. Anitschkow and others reported that cholesterol-fed rabbits develop microscopic atheroma in 4–7 weeks, and gross lesions in 8–11 weeks (268). Apparently, therefore, atherosclerosis can be induced in the chick with greater ease and rapidity than in the rabbit.

In contrast to spontaneous atherosclerosis in the chick, the greatest incidence of lesions with cholesterol feeding is

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The importance of this problem arises from the fact that diffuse lipid infiltration of the aorta precedes and accompanies atherogenesis in cholesterol fed and stilbesterol implanted cockerels. Is this the first stage of atherogenesis? Is this pre atherosclerosis? Is the focal lesion derived from the diffuse lipid infiltration of the aorta? The proponents of the infiltrative theory answer these questions in the affirmative (55, 102, 268). Others disagree (6–8). Hence this is a significant unresolved problem in atherosclerosis research.

vestigating the possibility that this lipid in the spontaneous lesions of chicks commercially fattened for the market was deposited as a result of increased intake of dietary cholesterol, e. g., through milk feeding. In any case, the spontaneous lesion would appear to be primarily a fibrotic process, as originally suggested by Dauber (300). However, this problem of its initial morphology and pathogenesis merits further study, particularly in view of Wilens' recent report indicating that diffuse intimal thickening of arteries may be a fibroblastic proliferative reaction secondary to lipid infiltration of the intima (577) (cf. pages 13, 79).

In addition to the foregoing spontaneous atherosclerotic lesion in the abdominal aorta, hens frequently exhibit a lipid infiltrative alteration of the elastic aorta. This is a change seen in roosters only rarely and minimally (292, 300, 311). Grossly this change is sometimes discernible as a bright yellow, streaky or spotty change in the intima of unstained preparations. Frequently it becomes apparent only after staining with Sudan IV. In all cases the involved area is smooth, flat and unelevated.

Microscopically these areas present a quite different picture from the spontaneous lesions in the abdominal aortas of hens and roosters. No fibrosis or atheroma is seen. The change consists of a diffuse accumulation of lipid in the intima and media. In cockerels, this sudanophilic lipid is most commonly found in the inner one-third of the media. The lipid is both intracellular, in fibroblasts, and extracellular, dispersed in fine droplets between elastic fibers. Chaikoff, et al., could demonstrate no cholesterol in this lipid (292). Neither lipid-bearing foam cells nor cholesterol crystal clefts are demonstrable.*

*Insofar as atherosclerosis is defined as a pathologic process producing thickening of the vascular wall, this "lesion" in hens and cockerels is not truly atherosclerosis. Its significance for atherogenesis remains obscure. Although both hens and roosters may exhibit this change in the elastic aorta, neither develop gross atherosclerotic plaques spontaneously at this site →



PLATE 1

Early cholesterol induced intimal atheroma in the thoracic aorta in a cockerel fed 2% cholesterol-5% cottonseed oil mash for the first fifteen weeks of life Note sudanophilic lipophages and absence of intimal fibrosis, as well as freedom from medial involvement This is "pure" atheroma, apparently the initial stage of cholesterol-induced atherosclerosis (see text) Frozen section. Sudan IV, X 125

in the ascending aorta and aortic arch (elastic aorta of the chick). In the earlier stages, the lining of the elastic aorta grossly has a deep tannish-yellow, granular appearance, without definitely identifiable discrete plaques. Subsequently elevated nodules, raised longitudinal streaks and plateau-like plaques are superimposed on the granular deposit. With further prolongation of high dosage (2%) cholesterol feeding, the lesions become confluent. Marked sclerotic changes supervene, rendering the aorta stiff and brittle. Birds on experimental diet for 15–26 weeks frequently exhibit such severe involvement of almost the entire elastic aorta. Definite gross narrowing of the orifices of the brachiocephalic and coronary arteries, and of the aorta lumen itself, result from the presence of these atherosclerotic plaques.

The distribution of lesions is fairly typical and consistent. The sinuses of Valsalva usually show considerable involvement, whether the lesions are mild or severe. The arch of the aorta and the first part of the descending aorta are also extensively affected. Except in the more severe cases, the involvement tends to peter out in the lower part of the thoracic aorta. Lesions in the brachiocephalic arteries, which are elastic arteries, usually parallel those of the thoracic aorta.

Cholesterol feeding also leads to an increased incidence and severity of gross atherosclerosis of the abdominal aorta (299, 307–312). However, this effect on lesions in the muscular aorta tends to lag behind, compared with the intensified atherogenesis in the elastic aorta. The abdominal aorta lesions of cholesterol-fed cockerels include large, yellow, elevated longitudinal plaques resembling the spontaneous lesions of birds not fed sterol-rich diets. In addition small, elevated, pinhead-like nodules may be seeded over the abdominal aorta, particularly adjacent to the orifices of aortic branches. Further, irregular plaques resembling those seen in the thoracic aorta, as well as raised, rough, granular, yel-



PLATE III

Somewhat more severe cholesterol-induced athero sclerotic plaque involving intima and media of thoracic aorta, and exhibiting degenerating foam cells, atheromatous "abscess" formation, cholesterol crystal clefts, lipid accumulation in fibroblasts, and a nidus of calcification Chick fed 2% cholesterol 5% cottonseed oil mash for the first 29 weeks of life Frozen section, Sudan IV, X 125



PLATE II

More advanced cholesterol-induced plaque formation in thoracic aorta of a cockerel fed 2% cholesterol-5% cottonseed oil mash for the first fifteen weeks of life. An atheromatous "abscess" has developed. Intimal fibrosis has supervened. The media is compressed. Frozen section, Sudan IV, X 75.



PLATE V

Intimal lipophage plaque narrowing a small coronary artery Chick fed 2% cholesterol-5% cottonseed oil mash for the first 12 weeks of life Frozen section, Sudan IV, X 75.

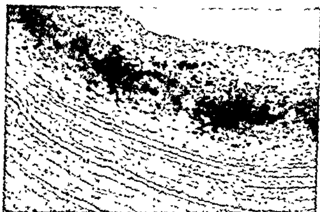


PLATE IV

Marked calcification and moderate intimal thickening in aorta in a chick fed 2% cholesterol-20% cottonseed oil mash for ten weeks and then fed plain mash for 15 weeks. This residual lesion is practically free of sudanophilic material (see text). Frozen section, Sudan IV, X 75.

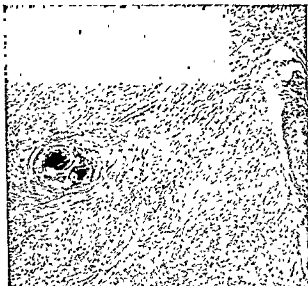


PLATE VII

Secondary calcification in foam cell plaques of small coronary artery in chick fed 2% cholesterol-5% cottonseed oil mash for the first 25 weeks of life Paraffin section, hematoxylin eosin, X 75



PLATE VI

Typical appearance of a hematoxylin-eosin stained preparation exhibiting cholesterol-induced atheromatosis of coronary artery, which is markedly narrowed by foam cells. Paraffin section, hematoxylin-eosin, X 125

low transverse lesions, are occasionally observed in the muscular aorta of birds with advanced atherosclerosis. Such involvement occurs with high dosages and prolonged periods of cholesterol feeding. In general, lesions in the iliac arteries parallel those in the abdominal aorta.

The cholesterol-induced lesions in the thoracic aorta exhibit a variable microscopic morphology, depending on their stage of development and severity. Early lesions consist of accumulations of large pale foam cells with small pyknotic nuclei. These foam cell plaques are of varying degrees of severity, from a single layer of cells to a veritable foam cell cushion, several layers deep. The endothelium overlying these foam cell cushions is intact. Sudan IV stains demonstrate fatty material in abundance within the subendothelial foam cells. The polarizing microscope reveals doubly refractile rodlike cholesterol crystals filling these cells. At this stage, special stains demonstrate no changes in the media and adventitia. This early lesion is "pure" atheroma. Apparently it is the initial lesion of cholesterol-induced atherosclerosis. The morphologic patterns of more advanced lesions are presumably the result of evolutionary pathologic processes secondary to atheroma.

In somewhat more advanced lesions, fatty material is seen in the media adjacent to the involved intima Orcein stains often demonstrate splitting of the elastica in such specimens.

In chicks fed high dosages of cholesterol (2%) for long periods (15–26 weeks), still more advanced changes are observed (309, 311). These processes include breakdown of foam cell plaques with total disappearance of foam cells or their conversion into "ghost" cells. Sometimes actual breakdown of the centers of the foam cell plaques occurs, with the formation of "abscesses" containing necrotic debris, fragments of nuclei, free fat, cholesterol crystal clefts and heavy deposits of calcium in granules or plates. Concomitantly,

from cholesterol-induced lesions only in their color, which may be bright lemon yellow or orange rather than cream.

Microscopically these lesions are typical atheromas, with fibroblastic intimal thickening, foam cells of the macrophage type, lipid-bearing fibroblasts, extracellular lipid droplets, and cholesterol crystal clefts. Birefringent crystals of cholesterol are demonstrable with the polarizing microscope. The aortic intima and media adjacent to these discrete, raised plaques are diffusely infiltrated with sudanophilic material.

In some estrogen-treated cockerels, lesions essentially similar to the foregoing are also present in the lower abdominal aorta, above the bifurcation. In addition, typical "spontaneous" smooth, ridgelike, longitudinal plaques are seen. Grossly they differ from the spontaneous lesions of untreated control birds only in their tendency to be lemon yellow in color. Microscopically they are found to contain considerable sudanophilic material in large globules, particularly in the deeper and central portions of the intimal plaques and in the media. These lipid pools are largely extracellular. Cholesterol is demonstrable by polarized light (292).

D. Coronary Artery Lesions

In addition to the changes in the aorta, brachiocephalic and iliac arteries, avian cholesterol-induced atherosclerosis extensively involves other segments of the vascular tree (299, 309, 311). Particularly in birds fed 2% cholesterol mash for 15–25 weeks, gross and microscopic atherosclerosis is found in the heart valves, endocardium and coronary arteries Similar lesions are also present in the blood vessels of the spleen, adrenal and thyroid glands, and in the main renal arteries. Further, involvement of the pulmonary arteries and systemic veins occurs, in association with a generalized organ lipidosis (xanthomatosis) (cf pages 135, 280).

Both gross and microscopic lesions of the coronary arteries are prominent in cholesterol-fed chicks (292, 299,

fibroblastic proliferation and hyaline and cartilaginous metaplasia proceed, and bone formation may even occur. In the heavier plaques, numerous fine vasa are seen on rare occasions communicating with the lumen. Occasionally endothelialized spaces resembling vasa are found; these may be filled with foam cells, loose and in clumps (311).

The ridgelike, smooth, longitudinal lesions in the lower abdominal aortas of cholesterol-fed chicks exhibit a pathologic pattern similar to the spontaneous lesion. There is localized subendothelial fibrosis and hyaline thickening. In addition, these fibrous plaques may show a greater degree of atheromatous change than the spontaneous abdominal lesion of plain mash-fed birds. Thus cholesterol clefts, foam cells and "atheromatous abscesses" become readily demonstrable. The possibility arises that these atheromatous changes are secondarily superimposed on a pre-existent fibrotic lesion We are currently investigating this problem. Other lesions in the abdominal aorta tend microscopically to be very similar to the cholesterol-induced plaques in the thoracic aorta.

In summary, then, cholesterol-fed chicks exhibit the spectrum of changes seen in atherosclerotic lesions in man, including foam cell plaques, necrosis and atheromatous abscesses, fibrosis and hyalinization, calcification, and cartilaginous and osseous metaplasia. Ulceration of atherosclerotic plaques with thrombus formation is the only lesion seen in man that we have not observed in the chick.

C. Estrogen-induced Atherosclerosis

The *morphology* of *estrogen-induced atherosclerosis* in cockerels is detailed in reports by Horlick and Katz (293), and Chaikoff, et *al.* (292). Grossly, lesions in these birds are seen in the brachiocephalic arteries, the upper thoracic (elastic) aorta and the upper abdominal aorta. They are typical intimal plaques of varying sizes, tending to differ

Stilbesterol-treated cockerels frequently exhibit microscopic accumulations of lipid in the media of coronary arteries. However, no other changes from normal are found (313) (cf. page 203).

Hitherto, most investigators of experimental atherosclerosis tacitly assumed that atherogenesis proceeds in different arterial beds based upon essentially similar biologic laws. Hence most workers confined their studies to the aorta, and interpreted their findings as valid for atherogenesis in the coronary, renal, cerebral and other arteries The results of a recent experiment in our laboratory demonstrate this to be a fundamental methodologic error (546). Pick, *et al.*, showed that estrogen exhibition prophylactically inhibits coronary atherogenesis in cholesterol-fed cockerels, whereas it is completely without effect on aorta atherogenesis (cf page 203). From these findings it may be concluded that different laws determine atherogenesis in the aorta and coronary arteries. In fact, re-evaluation of accumulated data suggests that this conclusion is valid with respect to atherogenesis in the thoracic as compared with the abdominal aorta. Hence workers in experimental atherosclerosis cannot confine their observations to the aorta alone

The importance of this methodologic point is highlighted by the fact that human morbidity and mortality result mainly from coronary and cerebral atherosclerosis. Studies of human material suggest that in *homo sapiens* as well, atherogenesis may proceed according to different biologic laws in different arterial beds. Therefore, the experimentalist, aiming ultimately to eliminate disease and death due to atherosclerosis in the arteries of vital human organs (heart, brain, etc.), must study atherogenesis in these vessels of his experimental animals. He must attempt to elucidate the basis for the different atherogenic responses of the several arterial beds to a given experimental regimen. In our labora-

309, 311, 312). Gross yellow-white plaques are frequently observed in the first few millimeters of the coronary arteries. Microscopic lesions include "pure" atheroma, with lipid laden foam cells almost completely occluding the vessel lumen, as well as more advanced lesions. The latter are calcified plaques with intimal thickening, foam cell cushions, crystalline cholesterol, necrotic foci, compression of the adjacent media and apparent penetration of lipid into the media. In no specimen have we observed disruption of the endothelium. Lindsay and Chaikoff report essentially similar observations on cholesterol-induced coronary atherosclerosis in chicks (313). Contrary to Paterson, *et al.* (314–316), Lindsay and Chaikoff agree with our conclusion that these lesions are primarily intimal in origin.

In addition to the foregoing lesions, cholesterol-fed chicks also exhibit diffuse lipid infiltration of the intima and media of coronary arteries without foam cells, fibrosis or other changes. On occasion foam cell accumulations in the media are seen as an exclusive alteration.

Lindsay and Chaikoff also studied spontaneous and stilbesterol-induced lesions in the coronary arteries of cockerels (313). The only spontaneous lesion they were able to identify was a small intimal fibrous plaque, entirely free of lipids. In their opinion the so-called spontaneous focal degenerative medial lesion described by Paterson, *et al.* (314–316), is an artefact. However, at the 1950 meeting of the American Society for the Study of Arteriosclerosis, Paterson presented substantial histologic evidence demonstrating the presence of this lesion (317). Recent work by Paterson and Cottral indicates that this lesion may not be a manifestation of arteriosclerosis, but merely a part of the disease process of chick lymphomatosis (317a). It is not clear whether the intima overlying such a focal area of medial degeneration is a site of predilection for the formation of an atherosclerotic plaque in cholesterol-fed chicks.

Despite these differences, the conspicuous fact is that cholesterolized chicks exhibit the entire gamut of atherosclerotic lesions seen in man, except for ulceration and thrombus formation Moreover, these lesions are grossly and microscopically remarkably similar to those seen in man (298, 299, 309, 311, 312). Finally, as we shall detail later (page 280), such aorta and coronary artery lesions may be induced in chicks in the absence of gross hypercholesterolemia, organ xanthomatosis, and venous and pulmonary artery lesions. This pattern of lesions and lipemia, effected by feeding chicks a ¼% cholesterol mash, closely resembles that often seen in man. This finding negates frequently advanced criticisms (11) of the significance of experimental cholesterol-induced atherosclerosis for the pathogenesis of the human disease.

CAN QUANTITATIVE RELATIONSHIPS BE DEMONSTRATED BETWEEN CHOLESTEROL AND ATHEROGENESIS?

In order to permit the study of factors influencing avian atherosclerosis, it became essential to quantitate the interrelationships among amount and duration of cholesterol feeding, degree of hypercholesterolemia, and incidence and severity of atherosclerosis. Toward these ends, Horlick and Katz accomplished three series of experiments lasting 5, 10 and 15 weeks respectively (312). Each series consisted of five groups of 12 chicks each (Table XXX).

In order to permit quantitative evaluation of lesions, a system for gross grading of aortas for atherosclerosis was devised. Gradings ranged from 0 to 4, based on extent and character of gross lesions Throughout experiments in this department, aortas from different groups are prepared for grading by technicians, and mixed indiscriminately. They are then examined consecutively as unknowns by at least two

tory, studies of the coronary arteries, as well as of the aorta, are now a routine part of every atherosclerosis experiment.

ARE EXPERIMENTAL AND HUMAN LESIONS MORPHOLOGICALLY SIMILAR?

The morphology of cholesterol-induced atherosclerosis in the rabbit has been repeatedly described in great detail. Recent advances have made available for comparative pathologic analysis the lesions of experimental cholesterol-thiouracil atherosclerosis in the dog (56, 318, 319). Many investigators have weighed the pathologic findings and compared them with lesions in man (4, 6–8, 28–31, 80, 102, 186, 267, 269, 284, 297). A review of the extensive literature on this aspect of experimental atherosclerosis is beyond the scope of this monograph. Rather we shall merely survey the problem in the chick. Typical lesions are shown in the Kodachrome photographs, illustrations 1 to 7.

In an early paper, Dauber and Katz called attention to the anatomical differences between the aortas of man and chick (298). The chick aorta and the large elastic arteries of the root of the neck are richer in elastic tissue than the corresponding human vessels. The change to the muscular type of vessel occurs in the descending thoracic and midabdominal aorta. The intima of the avian aorta and other arteries is very thin, consisting merely of endothelium and a fine layer of acellular fibrous tissue. Unlike the intima of adult human arteries, the chick intima lacks a subendothelial fibrous and a musculoelastic layer. Furthermore there is no well demarcated, thick elastic sheet forming an internal elastic lamina in either the aorta or the smaller arteries As Dauber and Katz noted, "Such differences in structure might be expected to modify the form and extent of lesions without affecting the underlying process, since in all other respects avian and human blood vessels are similar" (298).

lesions, approximate percentage area of aorta involvement, and character of plaques (e.g., degree of elevation and narrowing of the lumen, degree of evolution of lesions as indicated by gross fibro-calcific changes). In the main, grade is based on number and size of plaques. Brachiocephalic arteries, thoracic aorta, abdominal aorta, and iliac arteries are graded separately. In the final tabulation, respective weighted averages are determined for brachiocephalics and thoracic aorta, and abdominal aorta and iliacs (Fig. 10), these values being based mainly on the respective aorta segment grading. The grading scale ranges from 0 to 4. A single discrete plaque, small in size (e.g, a pinhead-like lesion) is graded ¼. Larger single lesions are graded ½ to 1. Presence of more than one lesion results in a minimal grading of 1. Depending on the number of lesions, their size, the percentage area of the vessel segment involved, the character of the lesion, grades of 1 to 4 are given. Grade 4 lesions involve almost the entire aorta segment, patently narrow the lumen, and grossly exhibit fibro-calcific changes.

Unquestionably this grading method is empirical and subjective. However, when employed *with the precautions detailed above* it is, in our opinion, quite reliable. Failure to apply these precautions opens the way to uncontrollable errors, and renders meaningless all careful physiological and biochemical quantitation utilized by trained investigators during long weeks of experimentation on atherosclerosis. The autopsy analysis is the culmination of such experimentation. Its controlled quantitation is indispensable for research in this field

By means of this grading method, Horlick and Katz were able to demonstrate a more than qualitative relationship between atherogenesis and cholesterol feeding (312). In general, incidence and severity of atherosclerosis were related to per cent and duration of cholesterol feeding (Table XXX). Under the circumstances of this experiment, this re-

trained observers, and graded. In our experience, these precautions are absolutely essential to minimize subjectivity and prejudice in evaluating atherogenesis. Lesions, if any, and gradings are recorded graphically on special forms (Fig. 10). More recently we have begun to survey the utility of grading aortas both before and after gross staining with Sudan IV.

As indicated in Figure 10, consideration is given in grading gross aorta specimens to such factors as number and size of

Experiment

Chick #

Exp. Diet

Weeks on Diet

Age

Sex

Death Date

Post Date

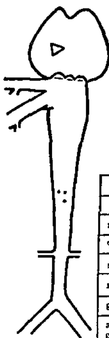
Cause of Death

Body Weight

Carcass Weight

Organ Weights

Gross Findings



	brachocephalics	thoracic aorta	abdominal aorta	iliacs
color				
raised?				
number				
% area				
size				
remarks				
grade				
average grade				

Fig. 10. Grading form.

lationship was only semiquantitative, and not linear. Study of the table reveals that with concentrations of cholesterol equal to 1%, and a feeding period of 10 weeks, an apparently maximal incidence and severity of atherosclerosis were obtained. Further increases of dietary cholesterol concentrations or prolongation of the experiment to 15 weeks had little intensifying effect on atherogenesis.

Concomitant study of the plasma cholesterol concentrations and weights of these various groups serves to clarify the foregoing findings (Figs. 11 and 12). In accordance with earlier procedures (298, 299), all cholesterol-fed chicks received 20% cottonseed oil in this experiment (312). The oil had a toxic effect on all these groups (cf. footnote, page 123). As indicated by the growth-development curves (Fig. 12), this toxic effect was somewhat enhanced in the presence of greater concentrations of cholesterol. Although exact data are not available, feed intake and weight change tended to parallel each other, i e., the birds getting the 4% cholesterol diet ate the least. Thus proportional increases in the percentage of cholesterol did not actually effect linear increments in cholesterol ingestion. Correspondingly, plasma cholesterol levels did not rise parallel with increases in percentage of dietary cholesterol above 1% (Fig. 11). Throughout the experiment, the most clearcut differences in plasma cholesterol level, and incidence and severity of lesions, were between the ½% group on the one hand and the 1–2–4% groups on the other (Table XXX and Fig. 11). This finding reflects not only the progressively and synergistically deleterious effects on appetite of 20% cottonseed oil plus increasing doses of cholesterol. It also may indicate an upper threshold to the amount of cholesterol which the chicken can ingest, assimilate and distribute from the gastro-intestinal tract (312).

In subsequent experiments Stamler, *et al.*, further clarified these interrelationships by eliminating the deleterious in-

TABLE XXX

Effects of Concentration of Dietary Cholesterol and Duration of Cholesterol Feeding on Incidence and Severity of Atherosclerosis—Horlick, L. and Katz, L. N. (312)

Cholesterol in Diet++ %	Thoracic Aorta						Abdominal Aorta					
	% Birds with Lesions			Average Grading of Lesions			% Birds with Lesions			Average Grading of Lesions		
	5 wks.*	10 wks.+	15 wks**	5 wks.	10 wks.	15 wks.	5 wks.	10 wks.	15 wks.	5 wks	10 wks	15 wks.
0	0	0	0	0	0	0	0	0	10	0	0	0.1
½	25	80	67	0.1	1.2	1.3	0	40	67	0	0.3	1.2
1	66	100	92	0 7	2.5	2.4	8	80	75	0.1	1.5	0.9
2	75	100	92	1.3	3 0	2 2	33	100	83	0.4	1.7	1.5
4	100	100	100	2 0	3.4	2 6	33	70	90	0 5	1.6	1.5

*Series 1.
+Series 2.
**Series 3.
++All cholesterol supplemented diets also contained [illegible]

lationship was only semiquantitative, and not linear. Study of the table reveals that with concentrations of cholesterol equal to 1%, and a feeding period of 10 weeks, an apparently maximal incidence and severity of atherosclerosis were obtained. Further increases of dietary cholesterol concentrations or prolongation of the experiment to 15 weeks had little intensifying effect on atherogenesis.

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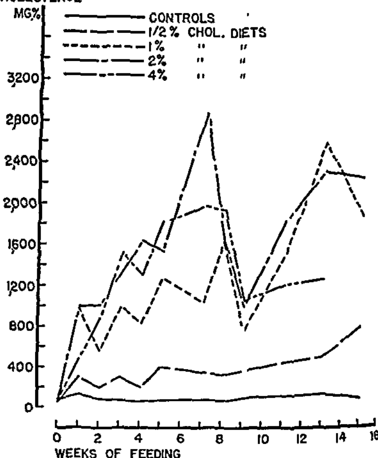


Fig. 11. Cholesterolemia in chicks fed various concentrations of cholesterol + 20% cottonseed oil—Horlick, L. and Katz, L. N (312).

fluence of 20% cottonseed oil. It was shown that ordinary crystalline, amorphous (320), and ether-dissolved cholesterol all are absorbed by the chick in the absence of cottonseed oil If dietary neutral fat is actually essential for absorption of ingested cholesterol (54, 57, 320, 321, 331), then the fat present in commercial chick starter mash (3–5%) is ade-

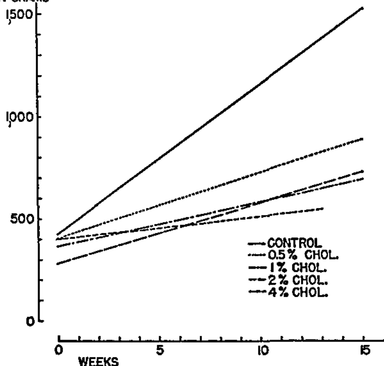


Fig. 12. Growth in chicks fed various concentrations of cholesterol + 20% cottonseed oil—Horlick, L. and Katz, L. N. (312).

quate for this need. The various types of dietary cholesterol without oil all induced a hyperlipemia similar in degree (307). Further, cholesterol with 5% oil was shown to be handled similarly to the foregoing preparations, insofar as resultant plasma lipid levels indicate. None of these cholesterol preparations, when fed in concentrations up to 2% in the diet, with and without 5% cottonseed oil, influenced feed intake or weight change. The birds followed the same course as controls (307–309).

These findings enable much closer quantitation. Using

such routines, we have found that with dietary cholesterol varying from ¼ to 2% plus 5% cottonseed oil, time of onset, incidence and severity of lesions, as well as degree of hyperlipemia, parallel percent and amount of dietary cholesterol intake (Tables XXXI and XXXII). This is true both with chicks placed on experimental diets at four to six weeks of age and when one day old (307–309).

We emphasize these points not merely for their intrinsic interest, in terms of the more than qualitative relationships among cholesterol ingestion, hyperlipemia and atherogenesis, but also because an additional basic methodological principle is involved here. In order to assess accurately the influence of various factors on experimental atherosclerosis, studies must be controlled with respect to each and all of the following factors: age, sex, initial weight, feed intake, final weight (322, 323). Season too is a factor, at least in the chick (309). Hence *groups of simultaneous controls, adequate in size, and with all the foregoing factors closely matched, are essential to every experiment.* Our experience leads us to concur completely with Firstbrook, who has recently emphasized these points (322, 323). Failure to control these factors jeopardizes experiments on atherosclerosis as fully as faulty biochemical analytical methods or an inadequate system for quantitating morphologic severity of lesions. This error may well have played a key role in contributing to the recording of misleading positive results reported by others in the literature, e.g., in experiments with lipotropic factors. Attention to these elements in an experiment will insure the degree of reliable quantitation in results predictable from the biology involved.

DO DIETARY FACTORS INFLUENCE EXPERIMENTAL ATHEROSCLEROSIS?

A. Undernutrition

In their original report on cholesterol-induced atherosclerosis in the chick (298), Dauber and Katz noted that their experimental diets of mash supplemented with cholesterol + cottonseed oil resulted in marked underfeeding and undernutrition. It was deemed essential to rule out the possibility that semistarvation, and not cholesterol feeding, was responsible for atherogenesis. In a subsequent project (299), therefore, Dauber and Katz studied this problem. Chicks were fed plain chick starter mash in the same amount as consumed by birds whose feed also contained cholesterol and oil. Thus the effects of underfeeding were assayed, separate and apart from the influence of cholesterol and oil. It was found that this undernutrition, established by underfeeding a balanced mash devoid of specific deficiencies, did not affect atherogenesis. These chicks had no lesions in the thoracic aorta. Some birds in this underfed group exhibited typical spontaneous lesions in the lower abdominal aorta. No significant difference was noted in incidence and severity of spontaneous atherosclerosis in underfed and *ad lib.* fed chicks.

In conclusion, therefore, limited dietary intake of a balanced mash does not result in atherosclerotic lesions of the induced variety. Moreover, such a dietary regimen has no inhibitory or intensifying effect on chick spontaneous atherosclerosis (299)

Dauber and Katz made the observation in the foregoing study that undernutrition may play a role in the development of hepatic lipidosis and hypercholesterolemia in cholesterol-fed birds. Several observations since amply confirm this impression. As already noted (cf. page 139), we have

such routines, we have found that with dietary cholesterol varying from ¼ to 2% plus 5% cottonseed oil, time of onset, incidence and severity of lesions, as well as degree of hyperlipemia, parallel percent and amount of dietary cholesterol intake (Tables XXXI and XXXII). This is true both with chicks placed on experimental diets at four to six weeks of age and when one day old (307–309).

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TABLE XXXII

Effects of Various Concentrations of Dietary Cholesterol on Incidence and Severity of Lesions, Using Diets Containing 0–5% Cottonseed Oil*—Stamler, J. *et al.* (307, 308)

Diet	% Birds with Lesions	% Birds with Lesions Grade 1 or >	Mean Grading of Birds with Lesions	Mean Grading for all Birds in Group
Plain Mash	0	0	0	0
¼ C—O	0	0	0	0
½ C—O	50	50	1.5	0.7
2 C	80	80	2.0	1.6
2 C—O	91	73	1.7	1.5

*Twenty weeks of age, 15 weeks on experimental diet
For plasma cholesterol levels, see Table XXXI.
For symbols, see Table XXXI

TABLE XXXI

Effects of Various Concentrations of Dietary Cholesterol on Cholesterolemia, Using Diets Containing 0–5% Cottonseed Oil*—Stamler, J. *et al.* (307, 308), Rodbard, S. *et al* (309)

Series 1		Series 2	
Diet	Plasma Total Cholesterol mg %	Diet	Plasma Total Cholesterol mg %
Plain Mash	78	Plain Mash	92
¼ C—O	150	¼ C—O	109
½ C—O	270	½ C—O	242
2 C	467	2 C	365
2 C—O	638	2 C—O	505

*All groups had similar rates of feed intake and weight gain.
Series 1 chicks placed on diet at 5 weeks of age, Series 2 at 1 day of age.
Bleedings in both series at 10 weeks of age.
¼ C—O = ¼% cholesterol + 5% oil added to mash.
½ C—O = ½% cholesterol + 5% oil added to mash.
2 C = 2% cholesterol added to mash.
2 C—O = 2% cholesterol + 5% oil added to mash

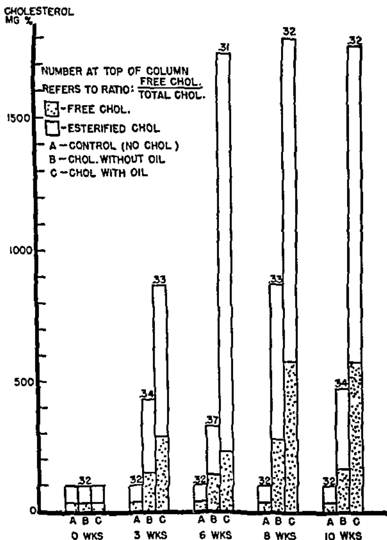


Fig. 14. Cholesterolemia in chicks fed 2% cholesterol diets with and without 20% cottonseed oil—Stamler, J., et al. (307).

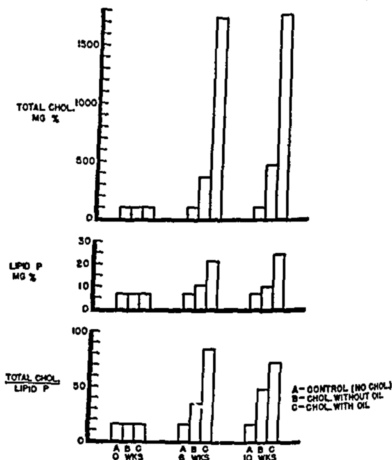


Fig. 13. Lipemia in chicks fed 2% cholesterol diets with and without 20% cottonseed oil—Stamler, J., *et al.* (307).

latterly abandoned the use of dietary regimens containing 20% cottonseed oil, in favor of 5% oil or no oil. With these latter routines, cholesterol-fed chicks exhibit normal feed intake, growth and development. They exhibit no signs of malnutrition. Chicks or a diet of mash supplemented with 2% cholesterol alone (2 C), or 2% cholesterol + 5% cottonseed oil (2 C-O) consume several fold greater quantities of cholesterol than birds receiving feed containing

turnover and metabolism, rather than decreased cholesterol absorption with smaller percentages of oil. Tracer studies are indicated to definitively clarify this point, as are studies of cholesterol absorption by chicks on a completely fat-free diet (119).

Rodbard, et al., in our laboratory have recently quantitated the relationships among decreased feed intake, hypercholesterolemia and atherogenesis in cholesterolized chicks (326, 326a). In one experiment, chicks were fed *mash containing either ¼% cholesterol plus 5% oil*, or 2% cholesterol. At each dietary level, one group of birds was permitted to eat *ad lib.*, another was given only 60–70% as much mash. The latter cockerels exhibited impaired growth and development. Decreased androgenic activity was indicated by pallor and smallness of combs (327). On their reduced dietary intake, these birds received less cholesterol than the controls given the same mash *ad lib.* Nonetheless the semistarved birds initially exhibited a more marked hypercholesterolemia than the controls (Table XXXIII). At the conclusion of the experiment, atherosclerosis was equal or greater in incidence and severity in the semistarved chicks, compared with the *ad lib.*-fed controls (Table XXXIV). Apparently, the underfed birds were unable to draw upon the elevated plasma lipids as a source of calories. On the contrary, in the face of undernutrition, dietary cholesterol would appear to be less readily disposable by the organism. Hence a more marked hyperlipemia supervenes and atherogenesis proceeds apace.

In another series of experiments, Rodbard, et al., approached this same general problem somewhat differently (328). Four groups of chicks were used in this further study. The first (Group 1) subsisted on a daily diet of mash supplemented with 2% cholesterol (2 C). The second (Group 2) was given this same feed (2 C) every other day,

2–10% cholesterol with 20% cottonseed oil, whose feed intake is markedly depressed. Despite their far larger cholesterol input load, the 2 C and 2 C-O cockerels exhibit significantly less marked hypercholesterolemia, hyperlipemia and organ lipidosis (Figs. 13–15, cf. also Fig. 11 and Table XXXI).

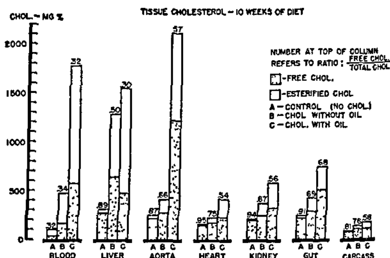


Fig. 15. Tissue cholesterol concentrations of chicks fed 2% cholesterol with and without 20% cottonseed oil—Stamler, J, *et al.* (307).

Some writers have attributed such a phenomenon to impaired cholesterol absorption in the absence of adequate neutral fat (54, 57, 80). We lean toward a different interpretation. Stamler, *et al.*, showed that the chick absorbs significant amounts of cholesterol when it is merely mixed with ordinary mash,* without any oil supplement (307, 308) (cf. 320, 324, 325). In our opinion, the lesser hypercholesterolemia of the well-nourished birds reflects nutritionally- and/or hormonally-influenced differences in cholesterol

*The commercial chick starter mash we use contains 3–5% crude fat.

TABLE XXXIII

Effect of Dietary Restriction on Cholesterolemia (in mg%) in Cholesterol-Fed Chicks—Rodbard, S. *et al.* (326a)

Weeks on Diet	¼ C—O Ad Lib	¼ C—O Restricted	2 C Ad Lib.	2 C Restricted
1	115	200	195	355
5	175	360	255	465
10	135	145	455	435
15	195	200	550	620
Mean	155	226	364	469

Symbols as in Table XXXI.

and was fed plain mash on the alternate days. The third (Group 3) also received the 2 C mash every other day, but went without any food on alternate days. Group 4 was made up of control birds subsisting throughout on plain mash ad *lib.* The effects of these regimens on cholesterolemia and atherogenesis after 20 weeks are summarized in Table XXXV. Both Groups 2 and 3 received no cholesterol on alternate days. Hence each had a total cholesterol ingestion that was approximately the same, and that was one-half the amount consumed by Group 1 birds. Despite these similarities, cholesterolemia and atherogenesis differed markedly in Groups 2 and 3. The latter birds, starved on alternate days, had far higher plasma cholesterol levels and aorta gradings (Table XXXV). Hypercholesterolemia and atherosclerosis in these cockerels were equal to or greater than that of the Group 1 birds, receiving approximately twice as much cholesterol. In contrast, the ingestion of plain mash on alternate days by the Group 2 chicks apparently played a decisive role in retarding hypercholesterolemia and atherogenesis. Overall adequate intake of a balanced diet apparently made a key contribution to the disposal of a load of dietary cholesterol. Whether this effect is mediated via dietary or endocrine factors, or both, remains to be elucidated (329, 330).

In all studies on experimental atherosclerosis, caution is indicated in relating the results of laboratory studies to the problem in man. We recognize that the foregoing projects, clarifying the effects of total body nutrition on cholesterol-induced atherosclerosis in the chick, deal with a situation rarely seen in man. Undernourished, semistarved people are seldom in a position to ingest significant quantities of cholesterol, since inadequate diets almost always lack animal fat. Under unusual circumstances, however, human beings may be subjected to a dietary situation similar to that of our experiments. Thus in a recent publication Malmros reported

Effects of Intermittent Starvation on Cholesterolemia and Atherogenesis in Cholesterol-Fed Cockerels—Rodbard, S. *et al.* (328)*

	Plasma Total Cholesterol mg %	% with Thoracic Aorta Lesions	% with Lesions in Whole Aorta	% with Thoracic Lesions Grade 1 or >	% with Whole Aorta Lesions Grade 1 or >	Mean Gross Grading—Birds with Lesions	
						Thoracic Aorta	Whole Aorta
Group 1 2 C—Ad Lib	334	73	100	55	91	1.8	2 9
Group 2 2 C—Alternate with Plain Mash	190	33	92	0	42	0.4	1.1
Group 3 2 C—Alternate with Starvation	483	100	100	100	100	2.8	3 6
Plain Mash	88	0	60	0	0	0	0.5

*Data obtained after 20 weeks of the experimental regimens.
Symbols as in Table XXXI.

TABLE XXXIV

Effect of Dietary Restriction on Incidence and Severity of Aorta Atherosclerosis—Rodbard, S. *et al.* (326a)

	Weeks on Diet	% with Thoracic Aorta Lesions	% with Lesions 1 or > Thoracic Aorta	% with Lesions in Whole Aorta	% with Lesions 1 or > Whole Aorta	Mean Gross Grading Birds with Lesions	
						Thoracic Aorta	Whole Aorta
Ad Lib. ¼ C—O	15	0	0	0	0	0	0
Restricted	15	20	20	60	20	1.0	0.6
Ad Lib. ¼ C—O	25	0	0	80	40	0	0 9
Restricted	18–24	50	33	50	33	1.9	2.2
Ad Lib. 2 C	15	80	60	80	80	1.8	2 0
Restricted	15	83	75	83	75	1.5	1.7

Symbols as in Table XXXI.

lesions are seen in 40–60% of birds of this age (Table XXXVI). Assuming the commercial chicks studied by Dauber (300) were subjected to a special feeding procedure for fattening, and hence had an excessive dietary intake, an incidence of 45% gross spontaneous atherosclerosis in these birds, together with their freedom from any lesions of the cholesterol-induced type, fails to indicate any effect on atherogenesis of such a dietary regimen. Since the dietary history of these birds is assumed, not accurately known, the foregoing deductions are tentative and subject to testing under carefully controlled laboratory conditions.

One other report in the literature deals with this problem. Wolffe, *et al*, indicate that forced-fed geese exhibit an increased incidence of both spontaneous and cholesterol-induced atherosclerosis (342). Evaluation of their findings awaits publication of their complete data.

Since obesity, the level of food intake in general, and cholesterol intake in particular have all been implicated as factors in human atherogenesis, further experimental studies are indicated on excessive dietary intake and atherosclerosis.

C. Neutral Fat Ingestion

In their initial report on cholesterol-induced atherosclerosis in the chick, Dauber and Katz utilized diets containing large quantities of the triglycerides, in the form of cottonseed oil (298). It became essential to determine the effect of this agent on atherogenesis. We have already had occasion to discuss the toxic effect on chicks of 20% cottonseed oil. Cholesterol feeding with this dosage of cottonseed oil leads to marked hypercholesterolemia (298, 299, 312). This hyperlipemic response is similar to that of semistarved cholesterolized chicks (326). We tentatively attribute it not to enhanced cholesterol absorption effected by oil, but to insufficiency of metabolic factors (dietary,

that during World War II the populace of Denmark subsisted on a luxus of cholesterol-rich foods, like eggs and milk, while intake of other components of a balanced diet was reduced (104). In contrast to other peoples of Europe, who in association with the undernutrition of the war years exhibited a fall in death rate due to atherosclerosis (cf. page 24), Denmark showed no decrease, and even a rise in this death rate (104). Although this finding cannot be regarded as more than suggestive, the possibility remains that in man the effects of exogenous cholesterol on cholesterolemia and atherogenesis may be regulated—via dietary and/or endocrine factors—by overall nutritional status.

B. Overnutrition

In contrast to these studies on semistarvation, only limited observations are available on the effects of forced feeding on experimental atherogenesis. The initial report by Dauber on spontaneous atherosclerosis in chicks offers some data on this problem (300). In that study she obtained "old roosters" (over one year of age) from a commercial processing plant. These birds had been farm bred for the market. Their dietary regimens were unknown. They may have been milk-fed and/or forced-fed for fattening. The former procedure would have exposed them to increased quantities of dietary cholesterol, the latter to excessive caloric intake.

The incidence of spontaneous aorta atherosclerosis in roosters recorded in this and other laboratories is summarized in Table XXXVI. Although some variability in incidence of spontaneous atherosclerosis is apparent, the findings are relatively consistent. Cockerels as young as 12 weeks may exhibit an incidence of spontaneous atherosclerosis as high as 20% (309). In general, the lesion begins to manifest itself macroscopically in roosters 12–20 weeks of age. Roosters 25–63 weeks of age have an incidence of spontaneous atherosclerosis as high as 71% (334). More usually

Dauber, D. V. and Katz, L. N.	298 299	Domestic Roosters	22–38 weeks	28%
Horlick, L. and Katz, L. N.	293	Domestic Roosters	25–33 weeks	40%
Stamler, J. *et al.*	308 339	Domestic Roosters	15–47 weeks	26%
Horlick, L. *et al.*	334	Domestic Roosters	26–50 weeks	71%
Stamler, J. *et al.*	341	Domestic Roosters	42–44 weeks	33%
Horlick, L. *et al.*	334	Domestic Roosters	51–63 weeks	50%
Kesten, H. D. *et al.*	304	Domestic Roosters	> 22 weeks	56%
Dauber, D. V.	300	Market Roosters	> 1 year	45%
Uchiyama, T.	305	Commercial	2 years	75%
Fox, H.	302	Wild Ground Fowl	> 11 years	5%
Fox, H.	301	Wild Ground Fowl	?	1.5%

TABLE XXXVI

Incidence of Spontaneous Aorta Atherosclerosis in Male Chickens of Various Ages

Author	Reference Number	Type Birds	Age of Birds	Incidence of Atherosclerosis
Rodbard, S. et al.	309	Domestic Roosters	12 weeks	20%
Horlick, L. and Katz, L. N.	312	Domestic Roosters	19 weeks	10%
Stamler, J. et al.	335	Domestic Roosters	20 weeks	0%
Rodbard, S. et al.	309	Domestic Roosters	26 weeks	40%
Horlick, L. and Katz, L. N.	311	Domestic Roosters	28–32 weeks	25%
Stamler, J. et al.	337	Domestic Roosters	30 weeks	33%
Stamler, J. and Katz, L. N.	340	Domestic Roosters	35 weeks	33%
Stamler, J. and Katz, L. N.	336	Domestic Roosters	40 weeks	50%

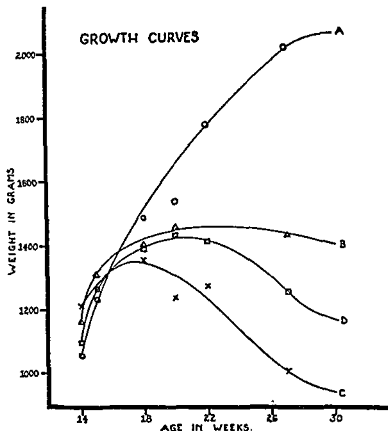


Fig 16. Effect of various dietary regimens on chick growth: A—controls; B—20% cottonseed oil mash, C—underfeeding, D—cholesterol + 20% cottonseed oil mash—Dauber, D. V. and Katz, L. N. (299).

cholesterol-induced atherosclerosis at autopsy. They exhibited an incidence and severity of spontaneous lesions essentially similar to that of the controls. The recent paper by Petersen and Hirst presented results in full agreement with the foregoing (662).

These experiments should not be interpreted as indicating that neutral fat ingestion is not a factor in human athero-

hormonal, enzymatic) essential for the disposal of absorbed cholesterol.

The further question arises: Does cottonseed oil influence atherogenesis in the absence of a cholesterol supplement in the mash? Dauber and Katz explored this problem in one of their early experiments (299). They placed four-week-old cockerels on a mash diet supplemented with about 20% cottonseed oil. Surviving birds were maintained on this diet for 32 weeks. These chicks exhibited impaired feed intake, growth and development (Fig. 16). Their feathers were dirty, greasy and matted Their combs and wattles were small and pale Their plasma cholesterol levels were slightly, but not significantly elevated (Table XXXVII). The plasma free/total cholesterol ratio was not significantly altered. No increase in incidence or severity of atherosclerosis was observed in the oil-fed birds. Three of 11 birds (27%) had spontaneous lesions of the abdominal aorta, compared with a 30% incidence (3 of 10) in the control group.

Recently Stamler, *et al.*, extended these observations to chicks fed plain mash supplemented with 5% cottonseed oil (341). At this level of oil, no impairment of feed intake, growth or development occurs, nor are there any signs of malnutrition or endocrine hypofunction. Mean plasma total cholesterol for the 15 week duration of the experiment was 85 mg.% (plain mash-fed controls: 108 mg.%). The plasma lipid phosphorus levels also coincided in control and experimental chicks (341). This finding, that neutral fat ingestion does not effect an alimentary hypercholesterolemia, is in accord with most previous observations on man and other animals (54).* Corresponding to these normal plasma lipid levels, the oil-fed cockerels showed no evidence of

*Some workers advanced data in support of the opposite conclusion (cf. 38, 331). The possibility remains that amount of neutral fat ingestion influences the plasma cholesterol containing lipoprotein micelle (cf. pages 32, 86, 99).

(enzymatic and hormonal) factors require experimental study and clarification.

D. Low Fat Diet

In several studies, Horlick, *et al.*, analyzed the effects of a specially prepared, defatted mash on both spontaneous and stilbesterol-induced atherosclerosis in cockerels (293, 334). Further, they investigated the influence of such a low fat, cholesterol-free mash on the regression of cholesterol-induced atherosclerosis supervening upon cessation of cholesterol feeding (311).

In the first study, 6–10-week-old cockerels were divided into two groups. Group 1 continued to receive regular commercial chick starter mash and tap water *ad lib.* Group 2 received the same chick starter mash from which the cholesterol and fat had been removed by repeated alcohol-ether extraction. The diets were made approximately isocaloric by the addition of sucrose to the latter mash; vitamins removed in the extraction procedure were also replaced (Table XXXVIII) (334). This diet given to the Group 2 birds contained 0.1–0.3% fat, as compared with 3.0–5.0% in the control regimen of regular chick starter mash. The extraction procedure reduced the cholesterol content from 60 mg.% to 0 mg.%, as determined by the Schonheimer-Sperry method (278), modified in this laboratory for analysis of tissues and feed (334).* Previous work has shown that an exogenous source of lipid is not essential for chick nutrition (345–347). The Group 2 cockerels exhibited normal feed intake, growth and development while subsisting on the defatted mash.

The experimental regimens were continued for 63 weeks, with chicks being sacrificed at intervals prior to that time. Plasma cholesterol levels were determined serially through-

*Other lipid analytical methods used in our laboratory are adapted from those of Man and colleagues (343, 344).

genesis. Clinically neutral fat and cholesterol ingestion are almost invariably combined—in contrast to the foregoing experiments. Considerable evidence exists that under such circumstances triglyceride influences cholesterol metabolism (54, 119, 152, 153). A recent experiment in our laboratory also indicates that under certain circumstances neutral fat

TABLE XXXVII

Effect of Dietary Cottonseed Oil on Cholesterolemia —Dauber, D. V. and Katz, L. N. (299)

Weeks on Diet	Plasma Total Cholesterol—mg %	
	Control Group	Cottonseed Oil Group
3	106	153
8	126	165
12	126	200
16	75	153
21	160	190

ingestion affects cholesterol metabolism and atherogenesis in the chick (341). This study will be presented in detail below (page 208), in the discussion of the pancreas and atherogenesis. Briefly, Stamler, *et al.*, demonstrated that the effect of cholesterol feeding in depancreatized chicks differs depending on the presence or absence of 5% cottonseed oil in the mash (341).

Such clinical and experimental observations indicate that a number of additional interrelationships among neutral fat, cholesterol, total caloric intake, and endogenous metabolic

out the experiment. These data are summarized in Figure 17. At the conclusion of the study, plasma lipid fractionations were done; the livers and carcasses were also analyzed for total and free cholesterol (Table XXXIX). Throughout the experiment, the plasma cholesterol levels of the chicks fed defatted mash (Group 2) were slightly (10–20%) higher than those of the controls. The other plasma

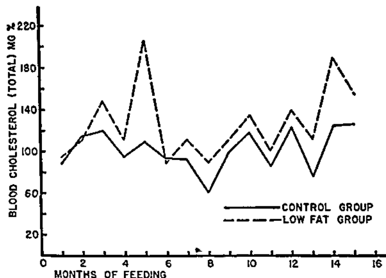


Fig. 17. Cholesterolemia in chicks fed plain mash (control group) and defatted mash—Horlick, L, *et al* (334).

lipid fractions were found to be similarly elevated (Table XXXIX). Liver and carcass cholesterol levels were not significantly different in the two groups. The two groups also had essentially similar patterns of fecal lipid excretion (Table XL). Thus it is apparently not possible to reduce plasma and tissue lipid concentrations below physiologically normal levels by excluding fats from the diet. Moreover, absence of exogenous cholesterol and neutral fat apparently had little effect on the hepato-hemato-enteric circulation of choles-

TABLE XXXVIII

Composition of Normal Chick Starter Mash (Plain Mash) and of Defatted Mash—Horlick, L. *et al.* (334)

Chick Starter Mash		Fat Extracted Chick Starter Mash	
Crude protein	18.0%	Crude protein	18.0%
Crude fat	3–5%	Crude fat	0.1–0.3%
Carbohydrate	54%	Carbohydrate	54%
Cholesterol	0.06%	Sucrose*	5%
		Cholesterol	0.00%
Vitamin		Vitamin*	
A	1,800 USP per lb.	A	1,200 USP per lb.
D_3	360 AOAC units per lb	D_3	180 AOAC units per lb
		E	30 mg per lb
		B (brewers' yeast)	9.08 gm. per lb.

Ingredients:
Ground corn, ground oats, ground wheat, wheat bran, wheat middlings, alfalfa meal, meat and bone scraps, dried buttermilk, soybean oil meal, steamed bone meal, calcium carbonate from limestone, salt 1.0 per cent, manganese sulfate 0.025 per cent, and potassium iodide, a trace.

*Added to mash.

TABLE XXXIX

Effect of Defatted Mash Diet on Plasma and Tissue Lipids—Horlick, L. *et al.* (334)

	Lipid P mg %	Phospho-lipid mg %	Total Cholesterol mg %	Free Cholesterol mg %	Cholesterol Esters mg %	Total Fatty Acids mg %
Plasma Lipids Control group	6 0	156	98	27	71	196
Plasma Lipids: Low fat group	6 7	174	125	43	82	243
Liver Lipids. Control group			307	272	35	
Liver Lipids· Low fat group			357	297	60	
Carcass Lipids Control group			110	100	10	
Carcass Lipids: Low fat group			107	97	10	

terol, insofar as plasma, tissue and fecal lipid concentrations indicate.*

The incidence of gross lesions in the aorta in the two groups is summarized in Table XLI. The gross morphology and location of lesions were typical of spontaneous atherosclerosis in roosters. Neither group exhibited induced lesions of the thoracic aorta. Spontaneous abdominal aorta lesions developed earlier in the control birds than in the chicks fed a low fat mash. However, in view of the variability in incidence of spontaneous atherosclerosis during this age period (cf. Table XXXVI, page 156), as well as the small number of chicks examined at this time (Table XLI), this difference is of questionable significance. It cannot be regarded as more than suggestive. Further, with prolongation of the feeding period beyond 50 weeks, the incidence of *gross atherosclerosis in the two groups* became approximately the same. The grading of the spontaneous lesions in the abdominal aorta was higher in the control chicks (1¼ vs. ¾).

From this experiment it is apparent that an exogenous source of lipid and cholesterol is not essential for spontaneous atherogenesis in the chick. If plasma lipids play a key role in this spontaneous atherogenesis,† endogenous sources continue to make available the necessary lipids in the absence of exogenous sources. Atherogenesis proceeds on the basis of normal plasma lipid levels derived solely from endogenous metabolic processes. However, absence of lipids from the diet may retard the onset and decrease the severity of this atherogenic process.

Application of this experiment to findings in man may well be held in abeyance.

In another experiment Horlick and Katz studied the effect

*Tracer studies are essential to obtain a more complete picture of these dynamic interrelationships.

†This is still an unsolved problem.

TABLE XLI

Effect of Low Fat Diet on Spontaneous Atherogenesis—*Horlick, L. et al* (334)

Duration of Feeding Period (Weeks)	Control Group			Low Fat Group		
	No of Chickens	No with Lesions	% with Lesions	No of Chickens	No with Lesions	% with Lesions
0–25	5	3	60	5	0	0
26–50	7	5	71	5	2	40
51–63	4	2	50	4	3	75
Total	16	10	63	14	5	35

TABLE XL

Fecal Lipid Excretion—Horlick, L. *et al.* (334)

	Total Fat mg %	Total Fat 24 hours	Fatty Acids mg %	Fatty Acids 24 hours	Lipid P mg %
Low fat group (4 chicks)	500	1,490 mg	358	1,070 mg.	trace
Control group (4 chicks)	595	1,465 mg.	361	885 mg.	trace

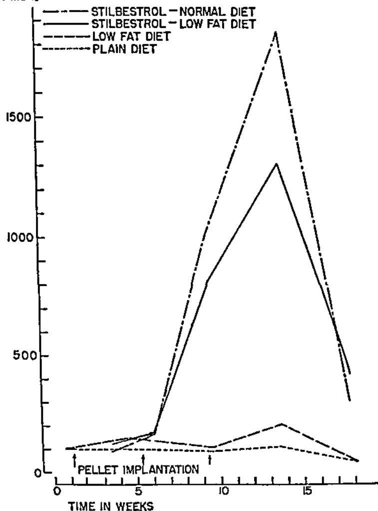


Fig. 18. Effect of stilbesterol implantation on cholesterolemia in chicks fed normal and defatted mashes—Horlick, L. and Katz, L. N. (293).

of a low fat diet on plasma cholesterol levels and atherogenesis in stilbesterol-implanted chicks (293). The effect on cholesterolemia is indicated in Figure 18. It is evident that the endogenous hypercholesterolemia induced by stilbesterol pellet implantation proceeds in the absence of exogenous lipid sources. The mean plasma total cholesterol concentration for the duration of the experiment was 561 mg % in the stilbesterol-treated chicks fed defatted mash, compared with 687 mg. % in their paired controls given regular mash. This difference is not statistically significant.

The failure of this experimental endogenous hyperlipemia to be greatly reduced by curtailment of exogenous fat parallels the limited success reported clinically in efforts to lower endogenous hypercholesterolemia (e.g., in primary hypercholesterolemic xanthomatosis) by dietary means. However, it contrasts with the observation of Keys that a fat- and cholesterol-free diet lowers hypercholesterolemia in such persons (119).

Corresponding to the persisting hyperlipemia, the stilbesterol-implanted chicks on low fat mash exhibited an incidence of atherosclerosis similar to their estrogen-treated paired controls fed regular mash (Table XLII). However, grading of stilbesterol-induced lesions was lower in the cockerels fed the defatted mash (Table XLII), suggesting a possible anti-atherogenic effect of this diet.

In a third study with defatted mash, the influence of this diet on regression of cholesterol-induced atherosclerosis was analyzed by Horlick and Katz in chicks following cessation of cholesterol feeding (311). In this experiment, birds were given a 2% cholesterol—20% cottonseed oil mash for 10 weeks. One group was then placed on defatted mash, the other on regular mash The therapeutic effect of the low fat mash was not consistently greater than that of the regular chick starter mash (Table XLIII, page 171).

In view of clinico-pathologic studies indicating that inani-

doxine-deficient diet induces intimal fibrosis (not atheroma) in monkeys, a species highly resistant to cholesterol-induced atherosclerosis (532). The initial lesion is characterized by an intimal accumulation of a mucinous substance with the metachromatic staining properties characteristic of mucopolysaccharides (cf. ref. 636, page 79). Cellular proliferation occurs and collagenous and elastic tissue fibers are formed (532a). These authors suggest that lipid accumulation is a later development. In a subsequent study, Greenberg and Rinehart observed that pyridoxine-deficient monkeys fed a low or moderate fat diet containing 1% cholesterol develop a greater hypercholesterolemia than normal controls (542). This supervenes although the cholesterol intake of the B_6-avitaminotic animals is ¼–½ that of the controls. After 65 weeks or longer of experimental feeding, neither group showed evidence of atherosclerosis; the arteriosclerotic lesions of pyridoxine deficiency were not enhanced by cholesterol feeding (542).

TABLE XLIII

Average Gross Grading of Cholesterol-Induced Aorta Lesions in Chicks Transferred to Defatted and Regular Mash—Horlick, L. and Katz, L. N. (311)

Weeks After Cessation of Cholesterol Feeding	Average Gross Grading of Lesions	
	Defatted Mash	Regular Mash
2–4	2 7	2.4
5–8	2.1	1 8
9–12	1 1	3 5
12–14	0 5	1.0

tion effects regression of atherosclerotic lesions in man (106), further experimental studies are indicated in the chick on the therapeutic effects of inadequate caloric intake (semistarvation), as compared with low fat and regular mashes fed *ad lib.* Further, in view of clinical and experimental data indicating only limited enteric ability to absorb cholesterol in the complete absence of dietary neutral fat, experiments are needed on lipid metabolism and atherogenesis in chicks fed fat-free diets plus cholesterol.

E. Vitamins

Little work has been done on vitamins and atherosclerosis, although these dietary substances play a key role in the intermediary metabolism of lipids (348). The few reports extant on vitamin D and atherosclerosis are contradictory Some workers believe large doses of vitamin D aggravate cholesterol-induced atherosclerosis in rabbits; others disagree (4, 349, 707).

Rinehart and Greenberg recently reported that a pyri-

TABLE XLII

Effect of Defatted Mash Diet on Cholesterolemia and Atherosclerosis in Stilbesterol-Implanted Cockerels—Horlick, L. and Katz, L. N. (293)

	Mean Plasma Total Cholesterol for Duration of Experiment mg %	% Birds with Lesions	Average Gross Grading of Lesions
Stilbesterol and Defatted Mash	561	60	1 2
Stilbesterol and Plain Mash	687	80	2.4

doxine-deficient diet induces intimal fibrosis (not atheroma) in monkeys, a species highly resistant to cholesterol-induced atherosclerosis (532). The initial lesion is characterized by an intimal accumulation of a mucinous substance with the metachromatic staining properties characteristic of mucopolysaccharides (cf. ref. 636, page 79). Cellular proliferation occurs and collagenous and elastic tissue fibers are formed (532a). These authors suggest that lipid accumulation is a later development. In a subsequent study, Greenberg and Rinehart observed that pyridoxine-deficient monkeys fed a low or moderate fat diet containing 1% cholesterol develop a greater hypercholesterolemia than normal controls (542). This supervenes although the cholesterol intake of the B_6-avitaminotic animals is ¼–½ that of the controls. After 65 weeks or longer of experimental feeding, neither group showed evidence of atherosclerosis, the arteriosclerotic lesions of pyridoxine deficiency were not enhanced by cholesterol feeding (542).

TABLE XLIII

Average Gross Grading of Cholesterol-Induced Aorta Lesions in Chicks Transferred to Defatted and Regular Mash—Horlick, L. and Katz, L. N. (311)

Weeks After Cessation of Cholesterol Feeding	Average Gross Grading of Lesions	
	Defatted Mash	Regular Mash
2–4	2.7	2.4
5–8	2.1	1.8
9–12	1.1	3.5
12–14	0.5	1.0

Flexner, *et al.*, found that vitamins B_1 and C are ineffective in preventing cholesterol-induced atherosclerosis in rabbits (350). Myasnikov observed vitamin A, thiamin and riboflavin to be without influence in cholesterol-fed rabbits, but he noted an incomplete, partial anti-hypercholesterolemic and anti-atherogenic effect of ascorbic acid (707). Dam observed that vitamin E does not modify aortic cholesterol deposition of cholesterol-fed rabbits or chicks (351). In contrast to these negative results, Morgulis and Spencer reported a significant rise in blood cholesterol in rabbits fed a vitamin E deficient diet (352), and Bruger noted that tocopherol markedly increases the cholesterol content of the aorta of rabbits fed cholesterol (353). Marx, *et al.*, found that neither avitaminosis E nor a large intake of vitamin E modifies plasma or tissue cholesterol levels in rats, or induces gross atherosclerosis (354). No studies have been carried out in our laboratory on vitamins and chick atherosclerosis.* Obviously the data of these few reports on vitamins and atherogenesis are fragmentary. This aspect of the over-all problem needs further exploration.

F. Proteins

The few experiments on proteins and atherogenesis have been recently reviewed by Hueper (4). In brief, several workers claimed that protein induces atherosclerosis in rabbits (355–359). Some of these experiments utilized "defatted" liver, testis, casein, or lean beef. None of these preparations were entirely free of cholesterol. Hence atherogenesis may have been cholesterol-induced. In one study, vegetable protein (oats) was used, with positive results (356). Others have failed to obtain lesions in rabbits fed vegetable protein (soybeans, gluten flour) (16–18, 357, 360)

*In all our experiments, with the possible exception of those using 20% cottonseed oil, chicks received apparently adequate amounts of vitamins in their diets.

or defatted animal protein (4, 361). Obviously further experimentation is needed, preferably in omnivorous species, to clarify the reasons for such contradictory findings and to elucidate the relationship (if any) of protein to atherogenesis. In our laboratory no projects have been undertaken to date on this problem.

G. Lipotropic Factors

Among dietary factors possibly influencing human and experimental atherosclerosis, either prophylactically or therapeutically, greatest attention has focussed on lipotropic factors. At least three observations led to the hope that these factors might be useful against atherosclerosis. First, it was noted that experimental cholesterol-induced atherosclerosis is usually associated with a fatty liver. It was reasoned that lipotropic factors, by combating hepatic cholesterosis, may cure or prevent atherosclerosis Second, it was postulated that lipotropic activity may not be confined solely to the liver These agents may play a role in removing from the arterial wall lipids deposited there in the initial stages of atherogenesis. Third, it was noted that some of these lipotropic factors prevent fatty livers in depancreatized dogs. Diabetic humans often have demonstrable derangements in lipid metabolism, with hyperlipemia, hypercholesterolemia and hepatic lipidosis. Moreover, they are particularly prone to be victims of atherosclerosis. The possibility arose that lipotropic factors may be specific replacement therapy, correcting the faulty lipid metabolism, particularly in diabetics, and thereby retarding atherogenesis

In view of these theoretical possibilities, the vigor with which lipotropic factors have been investigated in clinical and experimental atherosclerosis is not surprising Unfortunately the results have been at best contradictory, and in reality frankly disappointing. A series of recent reports yielding negative results in the chick, the dog, and the rabbit

are particularly decisive in indicating that at least choline is of no value in the prophylaxis or therapy of atherosclerosis.

A review of the great mass of research done during the last decade and a half on lipotropic factors is beyond the scope of this monograph. The reader is referred to a number of excellent reviews (54, 362–366). Prior to 1950, at least 13 communications had appeared in the English literature on various lipotropic factors in experimental atherosclerosis (367–379). As already indicated, contradictory results were reported. Some workers claimed success in preventing or treating experimental atherosclerosis with lipotropic factors Others obtained completely negative results. Most of these studies were done in rabbits. In our department Sanders obtained inconclusive findings with lipocaic in cholesterolized rabbits (266). The reasons for these disparate findings were not apparent.

Despite such inconsistent results and the paucity of controlled clinical data* (185, 211, 376, 380–390), practitioners are being persuaded on the basis of results by a few investigators to prescribe costly preparations of lipotropic factors for the prophylaxis and therapy of human arteriosclerosis. From both scientific and socio-economic considerations, therefore, it is essential that the actual effects of lipotropic factors on atherosclerosis be clarified. Towards this end, Stamler, *et al.*, undertook to study the possible prophylactic influences of choline and inositol on spontaneous, cholesterol-induced and stilbesterol-induced atherosclerosis in cockerels (308, 339). Further, this department recommended to the American Heart Association that it

*Davidson recently reviewed the limited clinical data available concerning [illegible] factors, cholesterolemia and atherosclerosis None of the studies [illegible] ol (663). Further, among [illegible], of S_f 10–20 lipoprotein [illegible] ered by administration of

take the lead in planning a nationwide, comprehensive, long term, definitive clinical study, of the type carried out on dicumarol in myocardial infarction (391).

In our study of lipotropic factors and cholesterol-induced atherosclerosis, we analyzed the effects of 1% choline + 1% inositol on birds fed three different concentrations of dietary cholesterol, ¼%, ½% and 2%. The exact dietary regimens are indicated in Table XLIV. A total of 96 cockerels were placed on these feeds at five weeks of age, 12 in each of the six cholesterol-fed groups and 24 in Group 7. The study was continued for from 15 to 35 weeks. Throughout this period, all experimental diets were well tolerated. The cholesterol-fed paired groups, with and without the choline-inositol supplement, exhibited feed intakes and rates of weight gain essentially similar to the controls (Group 7).

Complete plasma lipid fractionations were done serially

TABLE XLIV

Experimental Diets—Stamler, J. *et al.* (308)

Group	Mash %	Cholesterol %	Cottonseed Oil %	Choline %	Inositol %
1	94 5	0 5	5	0	0
2	92 5	0 5	5	1	1
3	98	2	0	0	0
4	96	2	0	1	1
5	94 75	0.25	5	0	0
6	92.75	0 25	5	1	1
7	100	0	0	0	0

throughout the experiment. All cholesterol-enriched diets produced hypercholesterolemia and hyperlipemia of varying degrees. Hypercholesterolemia was the principal alteration in the plasma lipid pattern. Phospholipids rose, but disproportionately less than cholesterol, so that the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ (C/P) ratio increased significantly. Cholesterol feeding induced no change in the ratio of free to total cholesterol in the plasma. In all cholesterol-fed groups, with and without a supplement of 5% cottonseed oil, total fatty acids, neutral fat fatty acids (calculated) and total lipid (calculated) tended to be elevated.

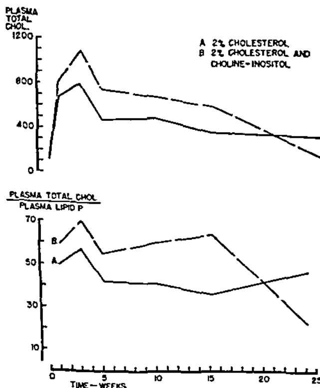
At all levels of cholesterol feeding, 1% choline + 1% inositol failed consistently to lower hypercholesterolemia and hyperlipemia. The lipotropic factors were without effect in ameliorating the minimal hypercholesterolemia consequent upon feeding ¼% cholesterol mash. The data indicate that during the initial weeks of the experiment addition of lipotropic factors to the diet actually aggravated hypercholesterolemia (Fig. 19).* At this time, hyperphospholipemia in the choline-inositol-fed chicks also tended to be more severe. However, the rise in phospholipid never kept pace with the rise in cholesterol. Thus exhibition of the phospholipid precursors choline and inositol (362–365) failed to elevate plasma phospholipid levels sufficiently to maintain a normal plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio in the face

*[illegible] and [illegible] offered a theoretical explanation that would ac-

liver, allowing free cholesterol to be excreted in the bile. However, biliary cholesterol is promptly reabsorbed in the intestines, back into the circulation, unless cholesterol absorption is prevented by extreme fat restriction or other measures to be considered" (57). Davidson also critically evaluated the theoretical considerations involved in the use of lipotropic factors in atherosclerosis (663).

of marked hypercholesterolemia. Likewise the lipotropic factors failed consistently to affect cholesterol-induced neutral fat hyperlipemia.

Fractionations of tissue lipids (liver, aorta, adrenal, gut, heart, lung, kidney, carcass) were accomplished serially during the experiment. Cholesterol feeding induced a lipidosis in all organs analyzed, most marked in the liver and adrenal



	% WITH LESIONS	AV GROSS GRADING	% WITH GRADING 1 OR >
A	89	1.5	67
B	100	2	67

Fig 19. Effect of choline and inositol on plasma lipids and atherosclerosis in the cholesterol-fed chick—Stamler, J, et al. (308).

TABLE XLV

Tissue Lipid Levels in Chicks Fed 2% Cholesterol (Group 3), 2% Cholesterol + 1% Choline + 1% Inositol (Group 4), and Plain Mash (Group 7) for 15 Weeks—Stamler, J. *et al.* (308)

Organ	Group	Lipid P mg %	Total Cholesterol *mg %*	Free Cholesterol *mg %*	Total Fatty Acids *mg %*	Total Lipid (Calc.) *mg %*
Plasma	3	10.4	375	140	877	1340
	4	9.5	508	205	968	1557
	7	7.1	70	33	384	514
Liver	3	127.0	3130	1006	5050	9267
	4	136.0	2024	1235	4190	7381
	7	84.5	287	277	5340	6354
Aorta	3	36 3	285	211	—	—
	4	34 9	279	205	—	—
	7	33 8	174	168	—	—
Adrenal	3	155 0	3230	570	5880	10440
	4	123.0	5020	564	7210	13185
	7	137 5	1157	515	5690	8029

Gut	3	72 6	716	448	1880	3221
	4	63 1	418	244	2830	3789
	7	50 6	246	236	4750	5431
Heart	3	95 0	220	176	—	—
	4	83 7	287	170	—	—
	7	83.8	155	147	—	—
Lung	3	91.0	688	600	1425	3059
	4	80 2	764	620	1570	3019
	7	71 4	477	468	3760	4849
Kidney	3	102.9	455	357	2180	3515
	4	96 9	668	349	2495	3832
	7	65.8	252	242	4910	5726
Carcass	3	28.3	170	133	3730	4142
	4	29 6	179	134	4510	4943
	7	20 8	116	69	6710	7004

(Table XLV). This lipidosis was minimal in degree in the birds fed ¼% cholesterol mash. It became more marked with increasing amounts of dietary cholesterol. Cholesterol, particularly esterified cholesterol, was the chief component of this tissue lipidosis. The increase in cholesterol concentration far exceeded the rise in phospholipid, which was often insignificant.

Choline and inositol had an incomplete lipotropic effect on the cholesterol-induced hepatic lipidosis (Table XLV and Fig. 20). This resistance (incomplete lipotropic response) of the cholesterol type fatty liver to choline and inositol is in accord with previous observations of several investigators (362–365).* The lipotropic factors were otherwise without consistent effect on the lipid concentrations of the other organs analyzed. They failed to reduce significantly or prevent aortic cholesterosis.

Gross autopsy findings in the aorta and great vessels of the seven groups in this experiment are summarized in Table XLVI. Obviously, addition of choline and inositol to cholesterol-enriched diets failed to reduce the incidence or severity of lesions in either the thoracic or abdominal aorta. Even in chicks fed ¼% cholesterol mash, exhibiting only minimal hypercholesterolemia and organ lipidosis (336), the lipotropic factors failed to prevent or alleviate atherosclerosis. If anything, the data suggest that exhibition of choline and inositol tended to increase the incidence and severity of atherosclerotic lesions.

In the companion experiment, Stamler, *et al.*, assayed possible prophylactic effects of 1% choline + 1% inositol against chick spontaneous and stilbesterol-induced atherosclerosis. Briefly, the lipotropic factors were without significant effect on feed intake, growth and development, or

*In contrast, Stamler, *et al.*, found that the hepatic lipidosis and cholesterosis of cholesterol-fed chicks is effectively prevented by exhibition of desiccated thyroid (392).

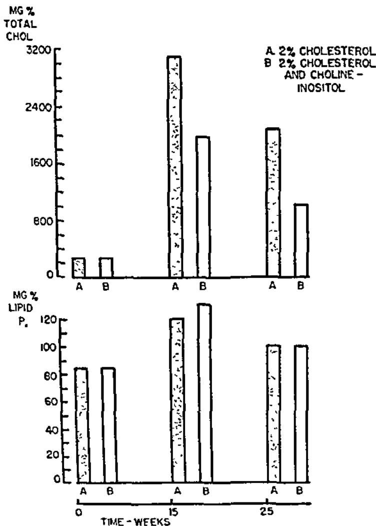


Fig. 20. Effect of choline and inositol on liver lipids in the cholesterol-fed chick—Stamler, J., *et al.* (308).

plasma and tissue lipids. Likewise they had no influence on aortic cholesterosis, or incidence and severity of either spontaneous or stilbesterol-induced atherosclerotic lesions (Figs. 21 and 22) (cf. also, studies with pancreatic factors, page 217).

Until recently, it was difficult to account for the disparity between the negative results obtained by others and ourselves, and the positive findings of several workers who studied the prophylactic effect of lipotropic factors against cholesterol-induced atherosclerosis. It appeared as if species differences might be accountable, since positive results (except for those of Herrmann (376–378)) were obtained in the rabbit. However, a number of investigators have recorded negative findings in this species as well.

Recently Firstbrook has reported a careful study in rabbits—with negative results (323). He took the precaution of force-feeding cholesterol and choline in gelatin capsules, rather than mixing them with the food. He thereby assured uniform dosage, and also avoided any possible depression of food intake due to the brackish taste of the drug. He deemed control of this factor essential since he previously found that the level of food intake influences experimental atherogenesis (322). Our findings are thoroughly in accord with this conclusion, as are those of Moses and Longabaugh (518).

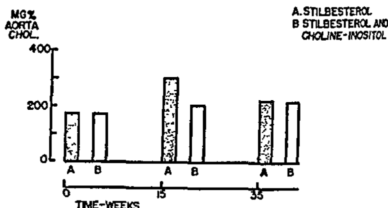
Firstbrook gave adult male rabbits 1 gram of cholesterol in capsules six times weekly. One group received 1 gram of choline chloride concomitantly. Experimental feeding was continued for a nine-week period. His results are summarized in Table XLVII, from his work (323). He found that degree of atherosclerosis increased with increasing blood cholesterol levels. The rabbits receiving supplemental choline showed no significant differences from the controls in average blood cholesterol level, body weight, or degree of atherosclerosis. Similar negative results with choline and/or

TABLE XLVI

Effect of Choline and Inositol on Cholesterol-Induced Atherosclerosis—Stamler, J. *et al.* (308)

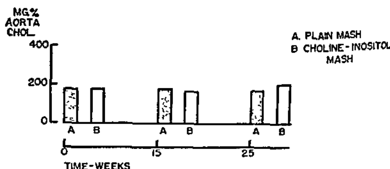
Group	Diet	Weeks on Diet	Number of Birds	Number with Lesions	% with Lesions	% with Grading Grade 1 or >	Av. Grading of Gross Lesions		
							Thor.*	Abd.*	Sum
1	0 5% Chol * + oil	15–25	12	6	50	25	0 3	1.0	1.3
2	0 5% Chol + oil + 1% choline + 1% inositol	15–25	11	8	73	55	0 8	1.3	2.1
3	2% Chol	15–25	9	8	89	67	1 0	0.5	1 5
4	2% Chol + 1% choline + 1% inositol	15–25	12	12	100	67	1.0	1.0	2 0
5	0 25% Chol. + oil	15–35	8	5	63	17	0	1.3	1.3
6	0.25% Chol. + oil + 1% choline + 1% inositol	15–35	11	6	55	38	0.5	1.5	2 0
7	Plain Mash	15–47	23	6	26	16	0	1.8	1.8

*Thor. is thoracic aorta, Abd. is abdominal aorta; Chol. is cholesterol.



	% WITH LESIONS	AV GROSS GRADING	% WITH GRADING 1 OR >
A	44	1.5	33
B	64	1	27

Fig. 21. Effect of choline and inositol on aorta cholesterol and atherosclerosis in the stilbesterol-implanted chick—Stamler, J., *et al* (339).



	% WITH LESIONS	AV GROSS GRADING	% WITH GRADING 1 OR >
A	26	1.5-1.7	16
B	28	1.5	17

Fig. 22. Effect of choline and inositol on aorta cholesterol and *spontaneous* atherosclerosis in the chick fed plain mash—Stamler, J., *et al.* (339).

inositol were obtained in carefully controlled rabbit experiments recently accomplished by Meyer, *et al.*, (664), Ellenbogen and Kendall (665), Moses (666) and Kellner (667) Duff and Meissner also found choline to be ineffective prophylactically or therapeutically, except when the level of dietary cholesterol is moderate (1 gram, three times a week) and the dose of choline is very large (668). Under the latter experimental conditions, a slight but definite retardation of atherogenesis was observed, unassociated with alterations in plasma lipids attributable to choline. Among the most recent papers, the only other reporting positive results is that of Dotti, *et al*, who found inositol to inhibit hypercholesterolemia in cholesterol-fed rabbits (669).

Davidson, *et al*, recently reported that choline chloride failed to influence the degree of atherosclerosis in dogs fed cholesterol plus thiouracil (393). The lipotropic factor was likewise without effect on serum or hepatic lipid levels.

As Davidson noted in his review on lipotropic agents in atherosclerosis, "Critical analysis of the published reports reveals that many of them must be rejected as inconclusive for various reasons" (663) The recent studies, which are free of the shortcomings of earlier experiments, almost uniformly yielded negative results Davidson cogently summarized methodologic lessons for atherosclerosis research to be learned from the confusing experiences over the years with lipotropic factors:

"Experimental arteriosclerosis, as produced in rabbits by cholesterol feeding, is subject to a large number of factors which influence the incidence and severity of the lesions. It is now recognized that even with all known variables controlled there is such marked biologic variation in experimental arteriosclerosis in rabbits that it is hazardous to use less than about 20 animals for each test regimen, with an equal number of control experiments limited to feeding the same quantity of cholesterol alone. In setting up an experiment

TABLE XLVII

Effect of Choline on Rabbit Cholesterol-Induced Hypercholesterolemia and Atherosclerosis—Firstbrook, J. B. (323)

Group	Mean Blood Total Cholesterol mg %	Initial Weight kg	Ratio of Final to Initial Weight	Mean Food Consumption per kg Body Weight grams/day	Degree of Atherosclerosis
Choline	185.8 ± 38.8*	3.254 ± .071	0.968 ± .048	31.4 ± 1.6	1.80 ± .58
Control	191.8 ± 25.6	3.349 ± .106	1.004 ± .044	35.2 ± 2.4	2.11 ± .51

*Standard errors throughout.

the control and test groups should be of identical strain, sex distribution, age and weight. They should be carried on experiment simultaneously for the same length of time Frequent serum cholesterol determinations should be made and the rates of weight gain should be followed. Neglect of any one of these requirements in the evaluation of a drug in the prevention or cure of experimental arteriosclerosis in rabbits leaves the results of the experiment open to question. The requirement of strain or heredity control is fundamental. Age has been shown to be an important factor in determining the ease with which arteriosclerosis can be produced in rabbits. Sex has been noted to play a role, in that some male rabbits seem to be rather refractory to the development of hypercholesterolemia and arteriosclerosis on this regimen. The initial weight and the rate of weight gain during the experiment are recognized as having an appreciable influence upon the arteriosclerosis. The thriving, weight-gaining rabbits seem to develop more arterial lesions than their lean brethren. *The necessity for simultaneous controls cannot be* emphasized too strongly, a requirement that is frequently neglected because it requires more in the way of facilities and personnel There is also the temptation to use a single control group for comparison with several successive test groups. In our own laboratory we have seen strictly comparable control groups of rabbits in successive years show significant differences in the incidence and severity of arteriosclerosis. This is most pronounced if the seasons happen to differ, because rabbits do not do well in summer Contemporary controls in the same animal quarters also serve as a check on such intangible variables as differences in commercial food lots and unrecognized intercurrent disease in the rabbit colony. These can inhibit the development of arteriosclerosis. Frequent serum cholesterol determinations serve to check the comparability of the control and test animals (when the test substance is to be used in a curative experi-

ment), or to reflect possible effects of the test substance upon the serum cholesterol (in the preventative type experiment). This latter use of cholesterol levels is hazardous because mere acceleration of the dietary cholesterol through the gastrointestinal tract by an irritative effect of the test substance can probably result in lower values. Finally, if all of these conditions have been complied with to achieve the ideally controlled experiment, at the end of such an experiment it usually is impracticable to perform the minimum of 20 control and 20 test animal autopsies within an insignificant interval of time.

"As a result of all these difficulties compromises have been made by all investigators with resultant invalidation of many of the experiments. Moreover, if a test group showed less arteriosclerosis than the control group, the test agent was likely to become the subject of a published report; if there was no difference between the groups, the investigator was generally inclined to dismiss the experiment as inconclusive. Custom seems to require much better data for negative reports than for positive ones.

"Most of what has been said of the design and interpretation of rabbit experiments applies in general to chicken and dog studies . . . [cf. pages 135–142—L N.K. and J.S].

"Most of the experiments cited by pharmaceutical firms in support of the use of lipotropic agents in arteriosclerosis are open to criticism on one or more of the above counts. When the literature is reviewed with these requirements for adequately controlled experiments in mind, there are only a few that escape rejection. In general, the results of even these experiments are suggestive rather than conclusive" (663).

To this statement one need only append a reiteration of the fact that the latest carefully controlled experiments with lipotropic factors yielded negative results.

In view of these consistent recent findings in three different species, we deem it worthwhile to reiterate state-

ments of Best (394) and Stamler, et al. (308), on lipotropic factors and atherosclerosis: "This claim for choline has no firm basis in fact and may serve as a false pathway which diverts energy from both the profitable study of atherosclerosis and the real realms of choline action" (394). ". . . *Neither on theoretical, nor experimental, nor clinical* grounds is there today a firm scientific basis for the widespread clinical use of costly lipotropic preparations in the prophylaxis and/or therapy of human atherosclerosis" (308).

H. Agents Possibly Inhibiting Enteric Absorption of Cholesterol

Endogenous cholesterol is secreted into the gut via the bile and the intestinal mucosa. Some of this endogenous cholesterol, together with some of the ingested sterol, is reabsorbed by the gut into the blood stream (54, 57). The possibility presents itself that this entero-hemato-hepatic circulation of cholesterol can be influenced exogenously. The intestinal disposal of cholesterol might be enhanced, plasma cholesterol levels lowered, and atherogenesis retarded.

Rodbard, et al., have been carrying on experiments on this problem in our laboratory (395). Utilizing a specially prepared aluminum hydroxide gel, it has been possible to decrease the hypercholesterolemia of cholesterol-fed cockerels and to retard atherogenesis.* This problem is being further explored.

Another experiment along similar lines was recently reported by Petersen (670). He compared levels of cholesterolemia in two groups of cockerels, one fed a mash supplemented with cholesterol and cottonseed oil, the other a mash containing cholesterol and cottonseed oil plus plant (soy) sterols. The plant sterols, which the animal gastro-

*Endogenous hypercholesterolemia induced by estrogens is not depressed by feeding this aluminum hydroxide preparation.

intestinal tract is incapable of absorbing (54), prevent the rise in plasma cholesterol induced by cholesterol feeding It was suggested that this effect may be due to interference by one or more of the mixed soybean sterols with enteric absorption of cholesterol (670).

In connection with this general problem of enteric absorption of cholesterol, cholesterol metabolism and the regulation of plasma cholesterol level (cf 54, 57, 555), Abell and Kendall recently reported two significant sets of observations (566, 671): Fecal excretion of sterols roughly parallels the level of dietary sterol in man, with wide individual variations. Constipation tends markedly to decrease the excretion of sterols. Secondly, dogs fed 10 grams of cholesterol per day excrete 2–4 grams of total sterol per day in the feces. Plasma cholesterol levels are only slightly elevated, ranging between 200 and 400 mg.%. When 1.2 grams of thiouracil are added to the diet of these animals, the rate of fecal sterol excretion is unaffected Nevertheless, plasma cholesterol levels rise to a mean of 1000 mg.%, and occasionally attain values as high as 5000 mg.%. It is clear from these observations that this marked hypercholesterolemia does not result from decreased enteric excretion of cholesterol, since this does not occur. Thiouracil-induced alterations in other aspects of cholesterol metabolism must be responsible (566, 671).

Clearly, further work is indicated on the relationship of enteric handling of cholesterol to cholesterolemia and atherogenesis.

WHAT ENDOGENOUS FACTORS INFLUENCE ATHEROGENESIS?

A. Thyroid Hormone

Since Anitschkow first induced experimental atherosclerosis by cholesterolizing rabbits, investigators have attempted to prevent, inhibit or treat the cholesterol-induced

lesions. The use of thyroid hormone, organic and inorganic iodides has yielded the best responses thus far. Katz and Dauber (38), Hueper (4), Gubner and Ungerleider (57), Katz, Stamler and Horlick (53), among others, have reviewed and summarized these results.

These observations have recently been extended and amplified by experiments with the chick in our laboratory (335, 337, 392, 406). In our initial study, Dauber, *et al.*, analyzed the effects of desiccated thyroid and potassium iodide (KI) in cholesterol-fed chicks. In three series of experiments, it was shown that desiccated thyroid significantly depressed the hypercholesterolemia of birds given diets supplemented with ½–2% cholesterol + 20% cottonseed oil (Fig. 23) (406). In contrast, KI did not lower plasma cholesterol levels. In accord with these plasma lipid patterns, desiccated thyroid significantly decreased the incidence and severity of cholesterol-induced atherosclerosis

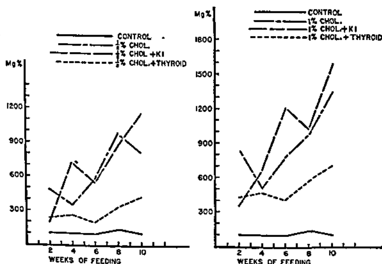


Fig 23 Effect of desiccated thyroid and of potassium iodide on plasma total cholesterol levels in cholesterol-fed chicks—Dauber, D. V., *et al.* (406).

(Table XLVIII). KI had no effect. These findings were consistent at all levels of dietary cholesterol utilized.

In one respect, these results were quite different from those obtained in the rabbit. Both species exhibited a similar lipid and atherogenic response to desiccated thyroid. However, inorganic iodides prevented the development of cholesterol-induced atherosclerosis in rabbits with an intact thyroid gland (407–411). Chicks on KI failed to exhibit this

TABLE XLVIII

Effect of Thyroid Feeding on Atherogenesis in the Cholesterol-Fed Chick—Dauber, D.V. *et al.* (406)

	% with Lesions Grade 0–1	% with Lesions Grade >1	% with Lesions Grade >3
Cholesterol Controls	33	67	22
Cholesterol plus Thyroid	48	52	11
Cholesterol plus KI	31	69	22

response (406). In attempting to account for this difference, note must be taken of the possibility that it is more apparent than real. Thus Moses and Longabaugh recently reported that KI had no effect on atherogenesis in young growing rabbits (679). (In our experiments young growing cockerels were used.) Rosenthal found that KI aggravated hypercholesterolemia and atherogenesis in cholesterol-fed rabbits (80). Obviously further work with iodides is needed.

In a subsequent study by Stamler, *et al.*, these observations were extended and amplified with respect to stilbes-

terol-induced atherosclerosis in cockerels (337). Plasma and tissue lipid fractionations were done in this study. During the first weeks of the experiment, exhibition of desiccated

[illegible]

treated birds eventually had similar plasma lipid levels, despite continued thyroid feeding in one (Figs. 24 and 25).

These findings are consistent with previous reports in the literature. Thus Fleischmann and Fried noted that thyroxine "neutralized" the effect of estradiol on serum cholesterol if equal doses of the two agents were given (412).

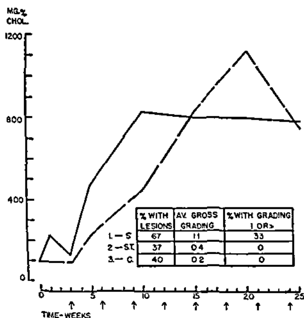


Fig. 24. Effect of [illegible]

experiment—Stamler, J., et al (337).

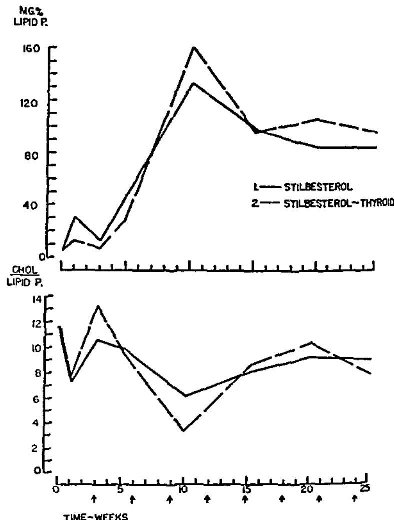


Fig. 25. Effect of thyroid on plasma phospholipids and C/P ratio in stilbesterol-implanted chicks (groups as in Fig 24)—Stamler, J, et al. (337).

When estrogen dosage exceeded thyroxine dosage, hyperlipemia ensued. This situation probably obtained in the stilbesterol-thyroid chicks during the latter weeks of our experiment, as a result of repeated stilbesterol pellet implantations.

TABLE XLIX

Mean Tissue Lipids of Stilbesterol (Group 1), Stilbesterol-Thyroid (Group 2) and Control (Group 3) Chicks—Stamler, J. *et al.* (337)

Group	Lipid P. mg %	Total Cholesterol mg %	Free Cholesterol mg %	Total Fatty Acid mg %	Total Lipid (Calc.)* mg %
Liver					
1	100	443	313	4490	5798
2	122	453	356	4330	5836
3	76.7	333	292	3518	4515
Aorta					
1	38.1	300	298	—	—
2	37 3	274	238	—	—
3	31 3	206	171	—	—

*Total lipid = phospholipid + total cholesterol + esterified cholesterol fatty acid + neutral fat fatty acid

This apparent predominance of stilbesterol over thyroid was also reflected in the liver and aorta lipid patterns at the conclusion of this study After 27 weeks of the experimental regimens, both estrogen-treated groups had a moderate aortic and hepatic cholesterosis and lipidosis, similar in degree (Table XLIX).

The similar aorta lipid findings contrast with the significant differences between the two experimental groups in gross pathologic grading for atherosclerosis (Fig. 24). The

stilbesterol-thyroid mash chicks had a significantly lower incidence and severity of atherosclerosis than the stilbesterol-plain mash birds. In fact, this latter group of cockerels and the control birds (Group 3—fed plain mash, without any hormonal supplement) exhibited a similar over-all incidence and severity of atherosclerosis (Fig. 24). Unlike these controls, however, the stilbesterol-thyroid mash chicks (Group 2) were not entirely free of thoracic aorta lesions, typically stilbesterol-induced in gross morphology.

It is difficult to account for the lack of correlation between biochemical and pathological findings in the two experimental groups. It may be related to the retardation of hyperlipemia induced by thyroid during the initial weeks of the study. However, even this early depression of hyperlipemia was never complete. In relation to their level of plasma lipids over the entire course of the experiment, the stilbesterol-thyroid chicks were remarkably free of lesions.

Similar results have been obtained in cholesterol-fed rabbits. Turner and Bidwell observed that KI prevented both hypercholesterolemia and atherosclerosis for several months (410). Thereafter the blood cholesterol rose, yet atherosclerosis did not develop. Likewise, in thyroidectomized rabbits fed cholesterol plus KI, thyroid hormone or thyroxine delayed but did not prevent hypercholesterolemia Even with hypercholesterolemia, however, these rabbits had very little atherosclerosis (410) More complete plasma lipid studies in such experiments, including C/P ratio and S_f level determinations, may serve to clarify the basis for this lack of correlation between cholesterolemia and atherogenesis (568, 681).

The possibility also arises that thyroid hormone exerts its effect on atherogenesis via mechanisms other than depression of hyperlipemia (4, 38, 57, 335, 337, 406, 413). Page and Bernhard found that an organic iodide* protected

*The di-iodide of ricinsterolic acid.

against cholesterol-induced atherosclerosis in rabbits, although it was without depressing effect on hypercholesterolemia and hyperlipemia (413). Similarly, both thyroid and thyrotropic hormones lower blood cholesterol, yet the latter favors cholesterol-induced atherogenesis (409, 410, 414, 415).* These and other findings (416) led some investigators to suggest that thyroid hormone affects atherogenesis via its influence on tissue accumulation of cholesterol, rather than via its effect on hyperlipemia. Others believe the hormone operates by altering vascular permeability (57, 417, 418). Lange has shown that thyroid hormone decreases intimal permeability (418). Iodides and thiocyanates have a like effect (417). All are inhibitors of atherogenesis (419, 420). Perhaps these substances prevent atherosclerosis by decreasing the permeability of the intimal endothelium to cholesterol or altering its capacity to phagocytose lipid? Further, Mardones, *et al*, recently suggested that thyroid exerts its effect on atherogenesis by inducing a rise in cellular cytochrome c (682).

Actually, the mechanisms whereby both thyroid and estrogenic hormones influence not only atherogenesis, but also lipid metabolism are inadequately understood Fleischmann and co-workers have concluded that both stilbesterol and thyroid exert their opposing effects on plasma cholesterol concentration by shifting sterol to and from blood plasma, rather than by altering the relationship between sterol accumulation and disposal (412, 421). Others, working with radioactive tracers, have obtained data tending to

*Preliminary studies by Stamler and Katz with thyrotropic hormone (TSH) yielded results indicating it to be without effect on [illegible]

[illegible] other experiments with TSH

support different conclusions. Stetten has recently suggested that hyperthyroidism leads to a disproportionate increase in hepatic degradation of fat (422). Lipid depletion results. Taurog, *et al.*, have shown that excised liver slices of stilbesterol-treated chicks form radio-phospholipid at an enhanced rate (423). They suggest that this is the source of stilbesterol-induced hyperphospholipemia. Stamler, *et al.*, recently obtained data indicating that chronic stilbesterol administration alters the organism's over-all lipid balance. In addition to hyperlipemia, the body total cholesterol, phospholipid and neutral fat increase (403).

Actually, the interrelationships between thyroid and estrogenic hormones extend beyond lipid metabolism, and apparently operate in many physiologic situations considerably different from those of the present experiment (424-427). Thus estrogens have been shown to suppress thyroid and plasma protein-bound iodine, probably via a pituitary action (428, 429).

Obviously elucidation of the mechanisms of thyroid effect on both cholesterol metabolism and atherogenesis awaits further clarification of these hormonal-metabolic relationships.

Towards this end, Stamler, *et al.*, recently undertook to determine whether the influence of thyroid hormone on lipids and atherosclerosis is due simply to hypermetabolism. We compared the effect of desiccated thyroid and of the hypermetabolism-inducing drug dinitrophenol on lipid metabolism and atherogenesis in cholesterol-fed chicks (335). The comparative effects of the two agents on plasma lipids are indicated in Figures 26 and 27. The birds fed cholesterol (Group 1) and cholesterol plus dinitrophenol (Group 2) had a similar pattern of hypercholesterolemia. The cholesterol-thyroid birds (Group 3) had consistently lower plasma cholesterol levels throughout the first 10 weeks of

the experiment. They had a value of about 300 mg.% (control: approximately 100 mg.%), less than one-half that of the other two cholesterol-fed groups.

All the cholesterol-fed groups except Group 3 (thyroid plus cholesterol) exhibited a hyperphospholipemia (Fig. 27). At no time, however, was this as marked as the hypercholesterolemia. Hence the plasma C/P ratio was elevated in all three groups. This elevation was considerably less marked in the birds fed thyroid plus cholesterol (Group 3, Fig. 27).

Data on aorta and liver lipids after 15 weeks of experimental feeding are presented in Figure 28. All three cholesterol-fed groups had elevated aorta cholesterol values. The increment was somewhat less in the thyroid-cholesterol group than in the other two experimental groups.

The liver lipid pattern was markedly different among the three experimental groups (Fig. 28). Groups 1 and 2 had greatly elevated hepatic cholesterol levels; this increase was somewhat more marked in the chicks fed cholesterol mash

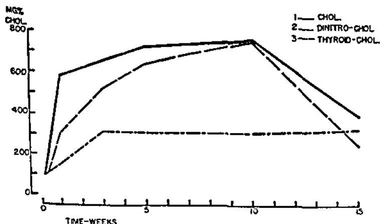


Fig 26 Effects of dinitrophenol vs. thyroid on cholesterolemia in cholesterol-fed chicks—Stamler, J, et al. (335).

(Group 1). In contrast, the Group 3 birds (thyroid-cholesterol mash) had normal liver lipid values.*

The gross pathologic findings are summarized in Figure 28. The control birds (Group 4) were free of gross lesions.

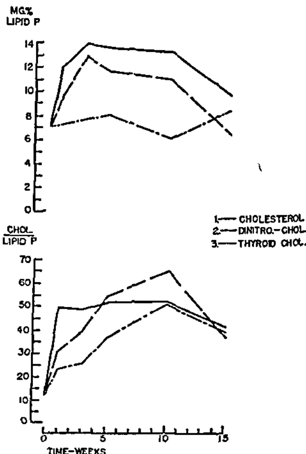


Fig. 27 Effects of dinitrophenol vs. thyroid on phospholipemia and C/P ratio in cholesterol-fed chicks—Stamler, J., et al. (335).

* [illegible] with

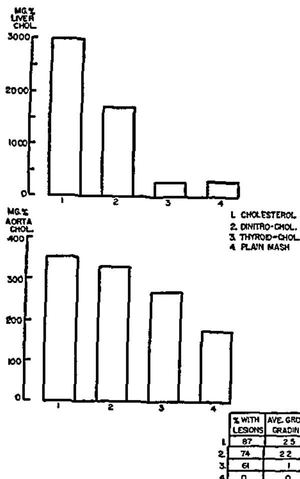
The chicks fed cholesterol mash (Group 1) and dinitrophenol-cholesterol mash (Group 2) had a similar incidence and severity of atherosclerosis. By contrast, the thyroid-cholesterol birds (Group 3) had significantly less extensive and severe atherosclerosis than the other two cholesterol-fed groups.*

In summary, this experiment demonstrates that the two hypermetabolism-inducing agents, desiccated thyroid and dinitrophenol, differ considerably in their effects upon lipid metabolism and atherogenesis. Unlike thyroid hormone, the pharmacologic agent dinitrophenol has little effect on plasma lipids in cholesterol-fed chicks This finding is in accord with results reported in a similar study on man (431). Further, dinitrophenol, unlike thyroid, does not exert a lipotropic action against cholesterol fatty livers. It has little or no effect on aorta cholesterol concentration in cholesterol-fed chicks. It is ineffective against cholesterol-induced atherosclerosis, whereas desiccated thyroid significantly lowers aorta cholesterol levels and decreases the incidence and severity of atherosclerosis (335)

In view of these findings, the effects of thyroid hormone apparently cannot be attributed solely to any generalized, nonspecific increase in energy exchange it induces. Rather than being nonspecific byproducts of increased metabolic rate, these actions of thyroid on cholesterol metabolism would appear to be effected via specific metabolic reactions involving hormone and lipid.

The specific pathways mediating these effects are obscure. Little is known concerning the intermediary biochemical reactions linking thyroid hormone and the lipids metabolically. The recent studies of Brown and Page are initial

*[illegible]



	% WITH LESIONS	AVE. GROSS GRADING	% WITH GRADING 1 OR >
1.	87	2 5	81
2.	74	2 2	58
3.	61	1	26
4	0	0	0

Fig. 28. Effects of dinitrophenol vs. thyroid in cholesterol-fed chicks aorta and liver cholesterol—Stamler, J., *et al.* (335).

steps in the direction of clarifying this problem (432, 433). They studied the effects of potassium iodide in cholesterol-fed thyroidectomized and unoperated rabbits. In intact cholesterol-fed animals smaller doses of iodide raised plasma cholesterol levels, larger doses had a depressing effect. In thyroidectomized cholesterol-fed rabbits both iodide dosages depressed hypercholesterolemia. Changes in hepatic choles-

terol ester fraction tended to parallel plasma total cholesterol. Presence of the thyroid was not essential for these "anti-cholesterol" effects of iodide (cf. ref. 408 and p. 192).

Brown and Page further correlated these findings with changes in various plasma iodine fractions (433). They found that the fraction of protein-bound iodine (PBI) containing thyroxine-like material* is not the mediator of the hypercholesterolemia-depressing effect of potassium iodide. This fraction apparently does not change with iodide and cholesterol administration. However, other fractions of the plasma iodine† increase in conjunction with the effect of iodide treatment on cholesterol metabolism.

Such experiments correlating lipid metabolism, thyroid and anterior pituitary function, inorganic and organic iodine levels, and atherogenesis need to be extended. A study of similar nature by Wolff, *et al.*, has yielded valuable information concerning the comparative effects of dinitrophenol and thyroid (434). It was found that dinitrophenol-induced hypermetabolism in rats is associated with a reduced PBI, in contrast to the elevated PBI of hyperthyroidism (435, 436).

The application of the latest methods of metabolic and endocrine research (61, 422, 437–441) to this aspect of the problem of cholesterol metabolism and atherogenesis should prove particularly fruitful.

B. Estrogen and Coronary Atherosclerosis

One of the key unsolved problems in human atherosclerosis is the sex differential in susceptibility to coronary atherosclerosis (cf. page 67). Hitherto, no experimental data giving any lead to the basis for this clinical phenomenon were available. However, a recent series of studies in our laboratory yielded significant new evidence bearing upon

*The alkali-washed, butanol-soluble fraction of PBI.

†The inorganic and butanol insoluble fractions.

this problem (405, 546, 683). The initial experiments were of the prophylactic type, in which cholesterol feeding and estrogen administration were combined. Two such experiments were accomplished (405, 546). In the first, two groups of one-day-old cockerels were placed on mash supplemented with 2% cholesterol plus 5% cottonseed oil. Simultaneously the group 2 chicks received daily intramuscular injections of 1 mg. estradiol benzoate.* In the second experiment, the identical procedures were followed, except

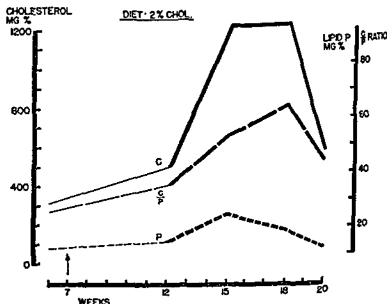


Figure 29 Plasma lipids in cockerels fed mash supplemented with 2% cholesterol + 5% cottonseed oil *C* is plasma total cholesterol; *P* plasma lipid phosphorus; *C/P* is the ratio $\frac{\text{Total Cholesterol}}{\text{Lipid P}}$; cf. Fig 30.—Pick, R , *et al* (546).

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* [illegible] both sub-
[illegible]
sion.

that the project was begun with 7 week old cockerels. The first experiment was terminated when the birds reached 12 weeks of age, the second at 20 weeks of age. The two studies yielded practically identical results which are summarized in Figures 29–31 and Table L. Estrogen administration had a marked influence on coronary atherogenesis in these cholesterol-fed chicks. It was remarkably effective in prophylactically inhibiting the development of coronary atherosclerosis. This morphologic finding was associated with significant estrogen-induced alterations in the plasma lipid pattern. By causing a marked rise in phospholipemia, without influencing cholesterolemia, the estrogen effected a significant fall in plasma C/P ratios to or toward the normal value of 12–14 (Figures 29–31 and Table L). Correlation of plasma lipid values and morphologic findings in the coronary arteries of individual chicks revealed a close association be-

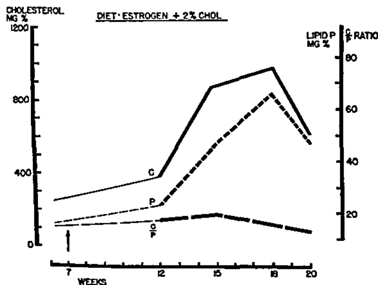


Figure 30. Plasma lipids in estrogen-injected cockerels fed mash supplemented with 2% cholesterol + 5% cottonseed oil. For symbols see Figure 29—Pick, R., et al. (546).

tween inhibition of coronary atherogenesis and degree of depression of C/P ratios toward normal. Thus all estrogen-treated, cholesterol-fed birds without coronary lesions had C/P ratios below 20 (Figure 31). In contrast to this estrogen inhibition of coronary atherogenesis, lesions in the aorta were completely unaffected.

As already indicated (cf. page 133), this latter finding demonstrates that various portions of the arterial tree (e g aorta vs. coronary arteries) differ in their atherogenic proclivities. Since a given prophylactic procedure (estrogen administration, in this case) prevents lesions in one region (the coronary arteries), without exerting this effect in another (the aorta), the possibility arises that atherogenesis proceeds in different parts of the arterial tree according to different laws. This is a conclusion of basic importance for atherosclerosis research (cf. page 133).

Further, the results of this experiment suggest (without proving) that a relationship may exist between coronary atherogenesis and plasma C/P ratios (cf. page 89). In this regard, preliminary data on plasma S_f patterns in the control and experimental groups of this experiment indicate that the plasmas of estrogen-treated cockerels contain significant concentrations of S_f 10–100 lipoproteins.* Further studies on S_f patterns in these birds are currently in progress, and conclusions concerning atherogenesis and specific classes of lipoproteins are held in abeyance pending their completion.

In a further experiment, R. Pick investigated the possibility that estrogens may also be therapeutically effective against previously induced experimental coronary atherosclerosis in cockerels (683). To test this, 50 chicks were fed a mash supplemented with 2% cholesterol and 5% cottonseed oil for five weeks. Ten chicks sacrificed at that time had

*These ultracentrifuge studies were accomplished in cooperation with Dr Lena Lewis and Dr. Irvine Page of the Cleveland Clinic Foundation, Cleveland, Ohio.

numerous, well-developed, lipid-laden, intimal plaques in the coronary arteries. The remaining chicks were divided into two groups; both continued to receive the same cholesterol-supplemented diet. One group also received daily 1 mg. injections of estradiol benzoate. After three weeks of this combined regimen, autopsy revealed a paucity of coronary lesions, with little or no lipid demonstrable in the remaining plaques. The residual lipid was present in the media, adventitia and perivascular connective tissue, while the intima was lipid free. In contrast, the other (control) group of cholesterol-fed cockerels, which received no injections, all showed numerous, extensive, lipid-laden coronary plaques.

These experiments demonstrate that estrogens are effective both prophylactically and therapeutically against cholesterol-induced atherogenesis in cockerels. They offer a fruitful lead to a possible basis for the relative immunity of women to coronary atherosclerosis, compared to men. Their

TABLE L

Effects of Estrogen Administration on Lipids and Coronary Atherosclerosis in Cholesterol-Fed Chicks—Pick, R. *et al.* (546)

	2 C—O*	2 C—O +E*	2 C—O+E Lesions Absent	2 C—O+E Lesions Present
Total Cholesterol mg %	895	742	758	701
C/P Ratio	47.1	15.9	13 6	21.5
Birds with Coronary Lesions %	100	33	0	100

*2 C—O is a diet of 2% cholesterol—5% cottonseed oil mash.
2 C—O+E is the same diet plus estrogen.

wider implications, including possible therapeutic significance in man, are being explored. (For a discussion on the influence of other gonadal hormones on experimental atherogenesis, cf. page 256.)

C. Pancreas

Until recently, experimental work on the interrelationships among the pancreas, the lipids and atherogenesis was meager. A few reports indicate the presence of vascular lesions in chronic diabetic dogs. Lukens and Dohan published autopsy findings on a dog with pituitary diabetes of five years' duration (442). This animal had subsisted on a lean beef diet. Atherosclerosis of the great vessels was absent, despite a moderate, apparently sustained hypercholesterolemia (210–396 mg.%).* Hueper cites his own observa-

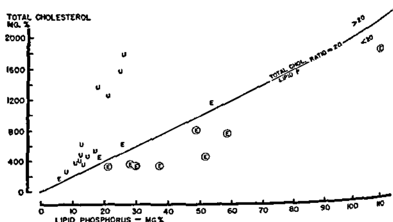


Figure 31. Plasma lipids and coronary artery findings in estrogen-injected (E and Ⓔ) and uninjected (U) cockerels fed mash supplemented with 2% Cholesterol + 5% cottonseed oil. All uninjected birds had coronary lesions. Four injected chicks had lesions (E); eight were completely free of lesions (Ⓔ). *All the latter* had C/P ratios less than 20 (Cf. text).—Pick, R., *et al.* (546)

*A positive finding was *intercapillary glomerulosclerosis.* This was the initial report of this lesion in an experimental animal.

tions on the occurrence of atherosclerotic lesions in the aorta and coronary arteries of dogs surviving complete pancreatectomy by several months (4). Fisher has reported similar findings (443). The data of Dragstedt, *et al.*, would tend to rule out the possibility that these are spontaneous atherosclerotic lesions, unrelated to removal of the pancreas (201, 444). They found an incidence of atherosclerosis in completely depancreatized, insulin-maintained dogs that was significantly higher than in normal controls. These animals had markedly fatty livers and normal or low plasma lipid levels (201). In contrast, partially depancreatized diabetic dogs, exhibiting moderate hyperlipemia and no hepatic lipidosis, remain free of atherosclerosis (201, 444). Allan has recently reported absence of lesions in such an animal with diabetes of 12 years' duration (445).

In commenting on the fact that other workers (446, 447) have not observed atherosclerosis in either completely or partially depancreatized dogs chronically maintained with insulin, Dragstedt and co-workers emphasized the possible significance of differences in dietary regimens employed. Thus many workers have kept totally depancreatized, insulin-treated dogs alive by feeding raw pancreas (446). Kaplan and Chaikoff gave no supplementary raw pancreas, choline or betaine, but utilized a diet low in animal fat (447–450) In contrast, Dragstedt, *et al*, fed a diet containing 25–45% animal fat without raw pancreas (201, 444). They suggested that this dietary lipid was a decisive factor in atherogenesis. An attempt to test this hypothesis by assaying the effect of cholesterol feeding on atherogenesis in the partially depancreatized dog yielded negative results (201) The problem has apparently not been explored further. To our knowledge data are not available on the effects of pancreatectomy on cholesterol-thiouracil-induced atherosclerosis in dogs (56).

Experimental work on these problems in the rat has been even more limited. Recently we were unsuccessful in an attempt to induce atherosclerosis in alloxan-diabetic rats by feeding cholesterol and cottonseed oil (266). Chute, et al, have reported negative results in similar attempts (451) (cf. also, references 684–686). These findings are in accord with many reports on the high resistance of this species to atherogenesis (4, 38, 53) (cf. discussion on species differences, p. 258).

A few investigations have been done on the rabbit Two studies report cataracts but no atherosclerosis in rabbits with longstanding alloxan diabetes (452, 453). Duff, et al. found that the visible lipemia of recently alloxanized rabbits was usually an evanescent phenomenon (402).* Despite the persistence of diabetes, plasma lipids tended to return to normal. Animals autopsied after several months were free of atherosclerosis. In a subsequent study, Duff and McMillan found that alloxan diabetes was without effect on the regression of cholesterol-induced atherosclerosis occurring in rabbits after cessation of sterol feeding (402).

choles-

37,

402). In contrast to findings in diabetic man, they made the unexpected observation that atherogenesis is retarded in some cholesterol-fed, alloxan-diabetic rabbits. McGill and Holman report a similar finding (454). Duff and Payne attempt to account for this apparent inhibitory effect of alloxan diabetes on cholesterol-induced atherosclerosis by relating it to the coexisting plasma lipid pattern They believe that the inhibitory effect is attributable to marked hyperphospholipemia and neutral fat lipemia, in association with hypercholesterolemia. These animals exhibit low plasma

*

caused

ol from

$\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ and $\frac{\text{total cholesterol}}{\text{neutral fat}}$ ratios, despite marked hypercholesterolemia. When such plasma lipid findings occur in nondiabetic animals, atherogenesis is again found to be retarded In their recent paper (287), Duff and Payne could demonstrate no correlation between atherogenesis and presence of "readily extractable" lipids* (286) in the plasma.

With respect to these experiments of Duff and Payne, Firstbrook offered the opinion that the phenomenon of inhibition of cholesterol-induced atherogenesis by alloxan diabetes ". . . might be associated with the characteristic emaciation of these animals, rather than with a specific effect of the diabetes" (322). However, Duff and Payne's paper indicates that this is not the case (287). Recently, Pierce, working with Gofman's group, repeated and confirmed Duff and Payne's work (678). Jones, *et al.*, summarized the data

demonstrated by Pierce in our laboratory studying the Duff type of alloxanized rabbit Duff has previously shown that feeding cholesterol to alloxan diabetic rabbits results in great elevations of serum cholesterol, to levels over 2,000 mg. per cent (Pierce actually has some with 5,000 to 10,000 mg. per cent), with minimal or no atherosclerosis developing. Pierce has shown that in such rabbits the cholesterol is transported in the form of molecules, primarily of the S_f 40–100 and higher classes, rather than in the S_f 10–30 class, as in the case of straight cholesterol feeding experiments. It appears, therefore, that high concentrations of molecules above S_f 50, with concomitant huge elevations of serum cholesterol, do not lead to atherosclerosis. In fact, in these experiments with alloxanized animals Pierce finds an inverse relationship

*Cf. page 98.

of total cholesterol with degree of atherosclerosis since, when the cholesterol is very highly elevated, it is primarily in molecules >50 S_f units which are apparently non-atherogenic and are not strongly associated with those lipoproteins that are atherogenic. It is an excellent illustration of the incorrect conclusion one is led to by the use of total serum cholesterol as a general index of atherosclerogenesis. At the same time these studies provide supplementary evidence of the differential significance of the various lipoproteins for atheroma formation. Throughout all types of rabbit experiments we have done, one common feature is evident, that is, elevation of the S_f 10–30 class of lipoproteins accompanies atherosclerosis. Total blood cholesterol correlates with atherosclerosis only in those particular types of experiments in which cholesterol elevation is associated with elevation of the S_f 10–30 class of lipoproteins" (556).

It would appear from these data that inhibition of atherogenesis in cholesterol-fed, alloxanized rabbits is related primarily to alterations in plasma lipoprotein patterns, rather than in C/P ratios.

To our knowledge, no investigator has reported development of atherosclerosis in any alloxan-diabetic species (dog, rat, rabbit, monkey, pigeon) subsisting on its normal diet (452). Additional studies are indicated in avian and mammalian species with alloxan diabetes of longer duration.

The over-all problem of the relationship of the pancreas to atherogenesis has been approached experimentally in one other way: With the discovery of insulin, it soon became apparent that this hormone alone was not adequate to maintain totally depancreatized dogs permanently (54). Insulin-treated, totally depancreatized dogs exhibited defects in lipid metabolism (fatty livers and hypolipemia), and failed to survive beyond a few months (54, 366, 447–450). Addition of raw pancreas to the diet corrected these metabolic defects and permitted chronic maintenance of the animals

(54). Subsequently lecithin and then choline were shown to be decisive nutritional factors in raw pancreas (54, 308, 455). Other essential dietary factors (pancreatic anti-fatty liver factors) were also identified, although not in pure form (362–366). These discoveries, in conjunction with the known defects in lipid metabolism and susceptibility to atherosclerosis of diabetic man, stimulated considerable research on the prophylactic and therapeutic value of pancreatic factors (dried whole pancreas, lipase, lipocaic, anti-fatty liver factors, lecithin, choline) in clinical and experimental atherosclerosis. We have already discussed the experiences with choline. Studies with lipocaic have also yielded contradictory findings (cf. page 174). To date this line of research has yielded disappointing results in both man and animals.

In our laboratory, these problems have been explored principally in the chick. We have found this avian species quite suitable for such studies, since it is highly susceptible to spontaneous, cholesterol-induced and estrogen-induced atherosclerosis, and is readily amenable to total pancreatectomy and alloxanization (341, 456). As our initial project, Stamler, *et al.*, undertook to determine the effect of pancreatectomy on plasma and tissue lipid levels, and on the three types of atherosclerosis in the chick (341). Two series of experiments were done. The experimental regimens are indicated in Table LI.

In accord with previous reports (457, 458), pancreatectomy was without sustained effect on plasma or blood glucose levels in the chick. Intravenous glucose tolerance tests on fed birds revealed essentially similar curves for normal and depancreatized chicks. None of the experimental regimens affected the blood glucose level or the glucose tolerance curves in pancreatectomized or control cockerels regardless of the degree of concomitant induced [illegible] lipemia.

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Plasma lipid findings are summarized in Table LI. Pancreatectomized chicks subsisting on plain mash alone (Group 1) exhibited normal plasma cholesterol and phospholipid levels throughout the experiment. These determinations yielded no evidence of a chronic disturbance in lipid metabolism in normally fed, depancreatized cockerels.

Operated birds eating a diet of mash supplemented with 2% cholesterol + 5% cottonseed oil (Group 3) exhibited a pattern of plasma lipid levels significantly different quantitatively from their paired controls.* Throughout the experiment in both series, the depancreatized birds responded to this diet with a far more severe hypercholesterolemia (Table LI and Fig 32). Both groups had only a moderate hyperphospholipemia, similar in degree. The plasma C/P ratio rose several fold, this value being significantly higher in the pancreatectomized chicks

In contrast to these findings, depancreatized birds fed a mash supplemented with 2% cholesterol (no cottonseed oil) (Group 5) had a plasma lipid pattern essentially similar to that of their unoperated paired controls (Groups 5 and 6, Table LI). They did not exhibit a hypercholesterolemia more marked than that of their paired controls. Apparently the addition of cottonseed oil to a cholesterol mash exerted a significant influence on the lipid metabolism of depancreatized chicks (cf. page 155). Among our studies, this is the first demonstrating a significant alteration in response associated with dietary administration of neutral fat.

Operated and control birds fed mash enriched with 5%

*Throughout the ... [illegible] ... are not attributable to variability in feed intake and nutritional state

TABLE LI

Effect of Pancreatectomy on Plasma Lipid Levels in Chicks on Various Regimens—Stamler, J. *et al.* (341a)

Group	Regimen	Mean Plasma Total Cholesterol for Duration of Experiment mg %	Mean Plasma Lipid Phosphorus for Duration of Experiment mg %	Mean C/P Ratio for Duration of Experiment
1	Panc.+—plain mash	110	7 6	14.5
2	plain mash	108	7.6	14.2
3	Panc.—2% C* + 5% oil	1310	16.7	78.4
4	2% C—5% oil	619	13 8	44.9
5	Panc.—2% C	507	10 0	50 7
6	2% C	430	11 2	38.4
7	Panc —5% oil	83	—	—
8	5% oil	85	—	—
9	Panc —Stilb **	514	56 2	9 1
10	Stilb.	544	68 8	7 9

+Pancreatectomized
*Cholesterol.
**Twenty five mg pellet of stilbesterol implanted subcutaneously every 3 weeks—groups 9 and 10 were fed plain mash.

patterns. Further, the paired groups had similar aorta cholesterol concentrations.

Findings in the aortas and great vessels are summarized in Table LII.* Despite normal plasma lipid levels in depancreatized chicks fed plain mash, these birds showed a slightly higher incidence and a considerably greater severity of atherosclerosis of the spontaneous type after 36 weeks on the diet.† Additional studies are indicated to test this phenomenon further. Our limited understanding of the pathogenesis of the spontaneous lesion renders difficult evaluation of this datum.

In conjunction with the more severe hypercholesterolemia and elevation of the C/P ratio, pancreatectomized cockerels fed mash supplemented with 2% cholesterol + 5% cottonseed oil (Group 3) had significantly more severe cholesterol-induced atherosclerosis. This pathologic finding could not be correlated with hepatic or aorta lipid values in these birds.

All other paired groups had essentially similar incidence and severity of atherosclerosis, in correlation with their like plasma lipid patterns.

Recently a further experiment was undertaken to ascertain whether the oral administration of various pancreatic preparations (whole dried pancreas, lipocaic, lecithin, pancreatin) prevented the inordinate hypercholesterolemic and atherogenic response of depancreatized cockerels to a cholesterol-oil mash (688). This project was repeated in three different series, with consistent results throughout (Table LIII). It is evident from this table that the addition of pancreatic factors to the mash of depancreatized chicks was without effect on cholesterolemia and atherogenesis under these experi-

*The number of chicks in Groups 9 and 10 surviving chronically was insufficient to permit pathologic evaluation of atherogenesis.

†However, no birds in Groups 1 and 2 developed lesions of the cholesterol-induced type in the thoracic aorta.

cottonseed oil alone had normal plasma cholesterol concentrations (Groups 7 and 8, Table LI).

No consistent differences were noted between depancreatized and unoperated cockerels in their plasma lipid response to repeated diethylstilbesterol pellet implantations (Groups 9 and 10, Table LI). Apparently the pancreas is not essential for the endogenous hyperlipemia induced by estrogens.

Unlike depancreatized dogs (54), our operated chicks did not develop a fatty liver on a plain mash diet. In general, pancreatectomized and control chicks of the several paired groups exhibited no significant differences in hepatic lipid

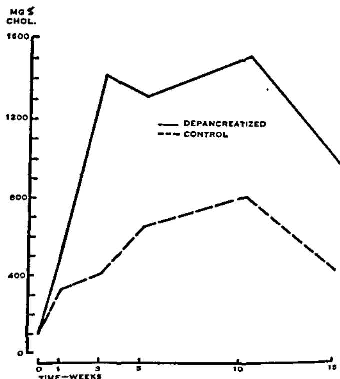


Fig. 32. Effect of pancreatectomy on cholesterolemia in chicks fed 2% cholesterol–5% oil mash—Stamler, J., *et al.* (3412).

TABLE LIII

Effect of Oral Pancreatic Factors on Cholesterolemia and Atherogenesis in Depancreatized Cholesterol-Fed Chicks—Stamler, J. and Katz, L. N. (688)*

	Total Cholesterol mg %	% Birds with Aorta Lesions	Mean Gross Grading of Aorta Lesions
Control— Depancreatized	1050	100	2.3
Experimental— Depancreatized + Pancreatic Factors	1057	97	2.5

*The data of this table combine the findings of the 3 series (see text). In 2 series, the diet was mash supplemented with 2% cholesterol plus 5% cottonseed oil; in 1 series, with 1% cholesterol plus 5% cottonseed oil. Birds were 7–8 weeks of age when placed on diet; experimental feeding varied from 5 to 9 weeks in duration. Pancreatic factors included lipocaic, pancreatin (pancreatic enzymes), whole dried pancreas, lecithin, 1% of each added to the experimental diet.

Effects of Pancreatectomy on Chick Atherogenesis—Stamler, *J. et al.* (341a)

Group	Regimen	% with Lesions	% with Grading 1 or >	Average Gross Grading of Birds with Lesions
1	Panc.—plain mash	46	46	1.6
2	plain mash	33	17	0.4
3	Panc.—2% C + 5% oil	100	92	3.6
4	2% C—5% oil	94	75	2.3
5	Panc.—2% C	83	67	1.8
6	2% C	88	88	1.9
7	Panc.—5% oil	67	0	0.3
8	5% oil	100	33	0.6

Symbols as in Table LI.

indicated by inordinate ACE-induced hyperglycemia in depancreatized or alloxanized chicks.

The results of experiments testing this possibility are summarized in Table LIV (459). Pancreatectomized chicks exhibited significantly greater and more prolonged hyperglycemic responses to ACE than controls.* These acute experiments demonstrated a relative insulin lack in the operated birds. Together with our experiments on cholesterol feeding, they suggest that chicks are not so fundamentally dissimilar from mammals (dog, rat, man) in carbohydrate and lipid metabolism as one might conclude from initial study of a pancreatectomized preparation.

These experiments with ACE are being extended to alloxanized birds. Preliminary data indicate that alloxanized cockerels exhibit a hyperglycemic response to ACE similar to that of depancreatized chicks. Studies are also in progress on the glycemic effects of individual glycocorticoids and of ACTH in the pancreatectomized chick. In this avian species, cortisone (compound E of Kendall; 11-dehydro-17-hydroxycorticosterone) has a minimal acute hyperglycemic effect, ACTH a moderate, and compound F of Kendall (17-hydroxycorticosterone) a significantly greater acute hyperglycemic effect (Table LV) (689).

Further studies indicate that prolonged exhibition of compound F to depancreatized cockerels induces a marked chronic hyperglycemia (cf. page 243) (690). Thus a chick preparation with a steroid-type diabetes is available for atherosclerosis research. Preliminary data indicate that when such depancreatized, compound F-treated chicks are fed cholesterol, a hypercholesterolemia develops far in excess of that exhibited by uninjected, depancreatized, cholesterol-fed birds. Despite this inordinate hypercholesterolemia, aorta

*ACE injection had no concomitant acute effect on plasma cholesterol or phospholipid levels of either control or depancreatized cockerels.

mental conditions (cf. also the negative results of experiments with lipotropic factors in intact chicks—page 173). Thus, oral pancreatic factors did not eliminate the inordinate hypercholesterolemic and atherogenic response of pancreatectomized birds to a cholesterol-oil mash.

These experiments demonstrated the existence of subtle defects in lipid metabolism in the depancreatized cockerel These defects were not apparent in the pancreatectomized chicks fed plain mash, for such birds exhibit normal plasma and hepatic lipid patterns. They were brought out particularly by a diet of mash enriched with cholesterol + cottonseed oil. On this regimen, depancreatized chicks showed an inordinate hypercholesterolemic and atherogenic response. Thus the lipid metabolic defects are associated with, and are apparently related to, intensified atherogenesis. Hence in the chick—as in man—the pancreas apparently influences the metabolism of lipids related to atherogenesis. This biologic similarity emerges despite initial data indicating that pancreatic deficiency in the chick and man has fundamentally different effects on lipid metabolism. Our findings would indicate that these apparent differences between man and chick are not necessarily qualitative ones, but rather at least in part quantitative.

In view of these findings, Stamler and Pick further undertook to elucidate whether similar subtle defects might not be present in masked form in the carbohydrate metabolism of the depancreatized chick (459). Mention has already been made of the fact that chicks chronically surviving pancreatectomy or alloxanization have normal blood glucose levels and glucose tolerance curves. The reasons for this remain obscure. The possibility arose that the gluconeogenic response to adrenal cortical extract (ACE) (54, 458) might overwhelm limited glucose regulatory mechanisms in such birds. A relative insulin lack might be demonstrated, as

indicated by inordinate ACE-induced hyperglycemia in depancreatized or alloxanized chicks.

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*ACE injection had no concomitant acute effect on plasma cholesterol or phospholipid levels of either control or depancreatized cockerels.

TABLE LIV

Hyperglycemic Responses of Depancreatized and Control Chicks to 10 Hourly Injections of ACE (1 cc)—Stamler, J. and Pick, R. (459)

	Plasma Glucose Pre-ACE mg %	Plasma Glucose 1 Hour Post-ACE* mg %	Plasma Glucose 20 Hours Post-ACE mg %	Plasma Glucose 40 Hours Post-ACE mg %
Control	223	403	251	198
Depancreatized	234	617	530	333

*One hour after last of 10 consecutive hourly injections.

TABLE LV

Acute Effect of Cortisone, Compound F and ACTH on Glycemia in Depancreatized Chicks*—Stamler, J. *et al.* (689)

Hormone	Procedure	Time of Bleeding after Last Injection Hours	Elevation of Blood Glucose* mg %
Cortisone	5 mg. every hour for 8 hours	1 24	35 0
Compound F	1.25 mg every hour for 8 hours	1 24	84 136
ACTH	3 mg. 3 times a day for 10 days	4 24	97 0

*All birds had normal blood glucose levels prior to injections.

atherogenesis is no more extensive, and coronary atherogenesis is significantly less marked, than in the control group (cf. page 246). Further studies are currently in progress with steroid diabetic chicks.

D. Hypertension*

Investigations into the problem of the interrelationships between hypertension and atherosclerosis [illegible] confronted by

S[illegible]

S[illegible]

sive effect,† and that experimental procedures utilized to produce hypertension, particularly renal hypertension, may induce renal hyperlipemia (183, 466–471). Further complications, often unknown, arise from the fact that we are dealing with interrelationships between two states, whose pathogenesis has in neither case been fully worked out. The investigator tends to feel somewhat inadequate in attempting to design controlled experiments in the face of such intangibles! He necessarily must proceed with caution!

The demonstration by Lenel, *et al.*, in our laboratory that salt feeding induces a significant blood pressure rise in chicks (472) made it possible experimentally to study the interrelationships between hypertension and atherosclerosis in this species Stamler and Katz recently compared atherogenesis in "salt" hypertensive and normotensive noncholesterolized cockerels (340). Chronic hypertension was induced in two ways: (1) by the substitution of 0.9% saline solution for drinking water (Group 1); (2) by the addition of up to 8% salt to regular chick starter mash (Group 2).

[illegible]

[illegible] hors [illegible] (4, [illegible] 64), [illegible] does [illegible] sive

A third group of 24 chicks, fed plain mash and tap water *ad lib.*, served as controls.

The experimental regimens had no effect on plasma cholesterol concentration. Blood pressure findings are summarized in Table LVI. In a majority of chicks, the elevations of blood pressure supervened after a few weeks of salt administration and persisted throughout the experiment.

The gross gradings for aorta atherosclerosis are summarized in Tables LVI and LVII. Lesions in all groups were located exclusively in the abdominal aorta and were morphologically of the spontaneous type. No gross lesions of the induced type were seen. The thoracic aortas and brachiocephalic arteries were free of lesions throughout.

Table LVI includes all birds in a given experimental group, regardless of blood pressure level. This over-all analysis fails to reveal clear cut differences between "salt" hypertensive and control chicks in incidence and severity of spontaneous atherosclerosis.

In Groups 1 and 2, 62% and 38% respectively of the chicks had terminal pressures greater than 180/150 mm. Hg. The autopsy findings of these birds exclusively are summarized in Table LVII. This analysis reveals higher incidence of aortic spontaneous atherosclerosis in the hypertensive chicks, compared with the controls In view of the known range of variability in the incidence of spontaneous atherosclerosis in control chicks (cf. Table XXXVI), these differences are of doubtful significance. This interpretation is supported by the fact that no consistent differences were recorded between hypertensive and control chicks in severity of spontaneous lesions (Table LVII).

In summary, "salt" hypertension had little or no effect in grossly intensifying spontaneous atherogenesis in cockerels. In the presence of normocholesterolemia throughout, no "salt" hypertensive chicks exhibited lesions in the thoracic or abdominal aorta of the cholesterol-induced type. Appar-

TABLE LVI

"Salt" Hypertension and Spontaneous Atherosclerosis: Mean Gross Gradings for Aorta Atherosclerosis, Based on All Birds in Each Experimental Group—Stamler, J. and Katz, L. N. (340a)

	Mean Blood Pressure mm. Hg	% Birds with Lesions	% Birds with Lesions Grade 1 or >	Mean Gross Grading of Lesions
Group 1 Saline	183/150	36	32	1.3
Group 2 Salt	181/148	20	0	0.5
Group 3 Control	163/138	33	19	1 0

TABLE LVII

"Salt" Hypertension and Spontaneous Atherosclerosis: *Mean Gross Gradings for Aorta Atherosclerosis; Groups 1 and 2 Include Only Birds with Blood Pressures > 180/150 mm. Hg.*—Stamler, J. and Katz, L. N. (340a)

	Mean Blood Pressure	% Birds with Lesions	% Birds with Lesions Grade 1 or >	Mean Gross Grading of Lesions
Group 1 Saline	191/156	54	38	1.3
Group 2 Salt	199/161	40	0	0.5
Group 3 Control	159/135	22	22	1.0

ently these lesions do not occur in the absence of an alteration in lipid metabolism, even in the presence of hypertension. This is in accord with the findings of this and other laboratories with several species (rabbit, dog, sheep, goat) (4, 80, 268, 460, 473–475).*

In contrast to our negative results in "salt" hypertensive *normocholesterolemic* chicks, several workers showed that hypertension intensified atherogenesis in *cholesterolized* rabbits.† Various forms of experimental hypertension have been utilized, including compression of the abdominal aorta above and below the renal arteries (268, 356, 476)‡ and maintenance of animals in an upright position (orthostatic hypertension) (477). The results have been consistent. Hypertension accelerated and aggravated cholesterol-induced atherosclerosis in rabbits.

The mechanism of this effect remains obscure. Some authors believe hypertension causes mechanical damage or ischemia of the arterial wall, rendering the vessel focally susceptible to lipid deposition. Others maintain that the elevated intra-arterial filtration pressure of hypertension increases the amount of lipid penetrating the intima, and thus enhances atherogenesis. Regardless of mechanism, the facts available from the few extant experiments support the view enunciated by Aschoff (102), and reiterated by Anitschkow (268), Rosenthal (80), Gubner and Ungerleider (57), Dock (478) and Faber (247). Anitschkow has

*Cf. our findings on spontaneous atherogenesis in DCA-treated, normal [illegible]

[illegible] showed [illegible] Gold [illegible]

[illegible] does [illegible]

rents.

summarized it as follows: ". . . . This etiologic factor (the increased cholesterin content of the organism) [is] the *exciting cause* of the disease. The increased blood pressure rather plays the part of a *predisposing* cause, inasmuch as it furthers the entrance of the lipoids into the arterial wall and their deposition in the intima" (Anitschkow's italics) (268).*

This interpretation of the relationship between hypertension and atherosclerosis is widely held at present. However, others have been advanced. Thus, Yater, *et al.*, recently suggested, "It may be that there is an hereditary predisposition to the two diseases or that there is a common etiologic factor" (35). In the present state of our knowledge this problem cannot be regarded as solved.

E. Pre-existent Vascular Damage

In the foregoing discussion, we noted that some workers attribute the effect of [illegible]

damaged arterial walls are the site of lipid deposition and atherogenesis. Virchow first advanced this theory in 1856 (75). He suggested that the lipid accumulations in atherosclerosis are degenerative local byproducts of a primary inflammatory proliferation of intimal fibroblastic tissue. Since then Aschoff (102), Hirsch and Weinhouse (86), and Duff (267), among others, have in one form or another adhered to the general concept that arterial cholesterosis and atherogenesis in man are processes occurring only secondarily, as superimpositions on previously damaged arterial walls (cf. page 78, et seq.).

Numerous attempts were made to test this concept experi-

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*Cf. the findings of Gofman, Lyon, Jones, *et al.* (152, 153, 538, 539, 556, 567), on S_f 10–20 lipoprotein molecule levels in hypertensive patients with and without evidence of atherosclerosis (page 109).

mentally. A host of noxious agents were tested for atherogenic effect, including bacteria, toxins, vasopressor and vasodepressor drugs, hormones, mechanical and thermal trauma, etc. The considerable literature reporting these studies was reviewed by Katz and Dauber (38) and Hueper (4). The findings can be briefly summarized. Various vascular lesions were experimentally produced by these methods. However, *atherosclerosis was never produced.* These negative findings stand in marked contrast to the positive results of cholesterol feeding experiments in rabbit, chick, dog, guinea pig, hamster and other avian and mammalian species.

Some of our experiments in the chick bear upon this problem: In a recent study by Rodbard, *et al.*, one-day-old cockerels were placed on a diet of chick starter mash supplemented with 2% cholesterol plus 5% cottonseed oil (309). This diet resulted in persistent hypercholesterolemia, without exerting any deleterious effects on nutrition, growth or development. During the first eight weeks of life the plasma cholesterol concentrations of these chicks ranged between 200 and 500 mg.%.

In the course of this experiment, birds were sacrificed at weekly intervals and their organs studied for gross and microscopic atherosclerosis. Gross cholesterol-induced lesions of the thoracic aorta and brachiocephalic arteries were observed in birds only five weeks of age. These chicks also exhibited microscopic atheroma of the coronary and splenic arteries (309).

It is apparent that these chicks had juvenile arterial tissue. Their vascular wall had not yet been subject to necrobiotic senescent alterations. Similarly, during their five weeks in the laboratory there was no indication in these healthy birds of the operation of pathologic stimuli (infectious, toxic, metabolic), noxious to the vessel wall, except for the hyperlipemia itself. The occurrence of significant gross atherosclerosis in these cockerels suggests, therefore, that

atherogenesis may proceed solely on the basis of deranged lipid metabolism, provided this is adequate in degree and duration.

Study of the control chicks of this experiment further enabled us to re-assess the time of onset of gross spontaneous arteriosclerosis in cockerels. In her original report, Dauber stated that the onset of gross atherosclerosis in roosters occurs at the age of five to six months (300). This latest study of Rodbard, et al revealed that gross spontaneous arteriosclerosis of the abdominal aorta may be seen in three-month-old control cockerels fed only plain mash, without cholesterol or cottonseed oil (309). Here again, young vessels are apparently susceptible to arteriosclerosis of the spontaneous variety.

The recent studies of Lindsay and Chaikoff (313) and of Paterson, Mitchell and Wallace (316) further delimit the role of tissue injury as a primary factor in chick atherogenesis. The data of the former workers indicate that cholesterol-induced atherosclerosis in the intima of chick coronary arteries is not superimposed on a pre-existent medial degenerative lesion. Similarly, Paterson, Mitchell and Wallace could not induce coronary arterial lesions or accelerate those of a spontaneous variety by injecting virulent staphylococci or their toxins. Nor could they reduce incidence of lesions by protecting birds against infectious disease (316). These observations are in general accord with experimental work in several species refuting infection-toxin "theories" of atherogenesis (4).

Based on these observations, we are in agreement with Anitschkow's theory that cholesterol is *primarily*, not secondarily, involved in experimental atherogenesis: "(1) The lipoidal accumulations in the arteries represent an infiltration of the latter with lipoids circulating in the blood (2) All subsequent hyperplastic processes are to be regarded as a reaction to the primary deposition of lipoids. (3) Choles-

terin plays the principal part in this deposition of lipoids in the arterial wall" (268).

The problem remains: Does injury to the vascular wall exert any influence on atherogenesis? A number of experiments bear upon this question. Anitschkow studied the combined effects of adrenalin administration and cholesterol feeding (268). He induced fibrotic plaques in rabbit arteries by repeated intravenous injections of adrenalin. He then fed these animals a high cholesterol diet. Atherosclerotic changes were superimposed on the adrenalin-induced arteriosclerotic lesions.* In similar experiments it has been further demonstrated that hypercholesterolemic rabbits develop intensified atherosclerosis at atypical sites, when these sites are the seat of arterial damage produced by such agents as thyroid hormone, posterior pituitary, etc. (4, 268).† Similar positive results were also recently obtained by Kelly, et al. (691) and by de Suto-Nagy and Waters (692) in studies on focal arterial injury and atherogenesis in the aorta, coronary and renal arteries of cholesterol-fed animals. Along these lines, Schlichter, et al., in our laboratory found that cholesterolized dogs develop intimal atherosclerotic lesions subjacent to areas of medial necrosis produced by adventitial cauterization (479). Further, spontaneous atherosclerotic plaques are apparently the site of predilection for lipid deposition when cholesterol is added to the chick's diet.

As Anitschkow pointed out, such findings establish "the etiologic significance of local lesions in the arterial wall" (268). However, recognition that "quite a number of different factors are involved in the etiology of atherosclerosis"

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*S[illegible] in man in Western [illegible] secondarily

† [illegible] bits using intravenous streptococcus toxin, oral ammonium hydroxide, artificial fever, intravenous peptone, anaphylactic shock and intravenous uric acid as vascular damaging procedures (357, 480).

(268) does not invalidate the fundamental conclusion: "If the insufficiency of the cholesterin metabolism is of a pronounced character, it may lead to the development of atherosclerosis even without any other concomitant causes" (268). This "combination theory" was formulated by Anitschkow as follows: "In human patients we have probably to deal with a primary disturbance of the cholesterin metabolism, which may lead to atherosclerosis even if the hypercholesterinemia is less pronounced, provided only that it is of long duration and associated with other injurious factors. . . ." (268).

F. Vasa Vasorum

Over the years, numerous workers have proposed that vasa vasorum pathology may be a factor in atherogenesis (4). In our laboratory, Schlichter, *et al.*, suggested that atherosclerosis developed subjacent to sites of arterial cauterization in cholesterolized dogs because the trauma diminished the rich vascularity of the animals' aortic walls (479). According to this concept, fixation of atherogenic lipids in the arterial wall is consequent upon inadequacy of the vasa vasorum.*

G. Adrenocorticotrophic and Adrenal Cortical Hormones

Considerable attention has recently been focussed upon the possibility that adrenal cortical steroids may induce vascular injury or may be otherwise involved in the pathogenesis of vascular scleroses. As we have already noted, clinical endocrine disorders characterized by excessive secretion of adrenal steroids (e g, Cushing's syndrome) tend to exhibit considerable atherosclerosis at autopsy. Experimentally, the mineralo-corticoid desoxycorticosterone has been

*Schlichter, *et al*, suggested that species differences in vascularization of the aorta (comparative richness of vasa vasorum supply) are factors in species differences in susceptibility to atherosclerosis (481, 482).

shown to induce hypertension and arteriolonephrosclerosis (but not atherosclerosis) (483-489).

Further, recent clinical studies with adrenocorticotrophic hormone (ACTH) and cortisone indicate that plasma cholesterol concentration is influenced by function of the anterior pituitary-adrenal cortical axis (240, 241, 584). The active part played by the adrenal cortices in sterol metabolism is further shown by their high concentration of cholesterol, their ability readily to discharge cholesterol, to synthesize cholesterol and to accumulate ingested cholesterol (308, 335-337, 490-493).

These facts alone, together with the growing chronic utilization of pituitary preparations and adrenal cortical steroids in clinical therapeutics, emphasize the need to clarify the relationships between these hormones and atherogenesis.

Recently Stamler, *et al.*, undertook two series of experiments on the effects of desoxycorticosterone acetate (DCA) on plasma lipids, blood pressure and atherosclerosis (spontaneous and cholesterol-induced) in chicks (404). DCA was given in daily doses of from 1-3 mg. for periods of 15 weeks, starting when the birds were five weeks of age. In the first series, the effects of this mineralo-corticoid were assayed on cockerels fed either plain mash or 2% cholesterol—5% cottonseed oil mash. In the second series, diets of plain mash and ¼% cholesterol—5% cottonseed oil mash were used. In addition, all groups of this second series received 1% sodium chloride in their mash, to enhance the possible hypertensive effect of DCA (483, 484, 489, 494-496).

In accord with previous observations in other species (497, 498), the chicks receiving DCA had hypoplasia of the adrenals. This was true of both cholesterol-fed and plain mash-fed birds receiving the mineralo-corticoid. This effect may be due to suppression of pituitary adrenocorticotrophic

hormone (ACTH) secretion by DCA (499).* Further, in accord with reports on other species (500–503), the cockerels receiving DCA exhibited polydypsia. This was observed in both Series 1 and 2, with and without a supplement of 1% NaCl in the mash. In a concurrent study, Stamler, *et al.*, found that this polydypsia was absent in DCA treated chicks fed a low sodium diet. Testicular weights and sizes tended to be smaller in the DCA chicks, as were comb size indices, indicating suppression of androgenic activity by DCA (404). None of these changes are attributable to nonspecific dietary and nutritional factors, since all groups exhibited essentially similar, normal rates of feed intake, growth and development.

In both series, DCA effected a significant increase in heart weight. In Series 1, the DCA chicks exhibited only slight, inconsistent elevations of blood pressure. In Series 2, the blood pressure increments were greater and more constant (Table LVIII)

In both experiments, DCA did not significantly alter either the normal plasma lipid pattern of chicks fed plain mash, or the degree of hypercholesterolemia in chicks given mash supplemented with cholesterol and oil. Apparently neither exogenous mineralo-corticoid administration nor the hypoadrenalism and hypoadrenocorticotrophism it apparently induced had any marked influence on plasma cholesterol levels in birds fed either plain or sterol-supplemented mash.

The findings in the aortas in Series 1 after 15 weeks of the experimental regimens are summarized in Table LIX In this series, DCA administration to chicks fed plain mash did not significantly influence the incidence or severity of spontaneous atherosclerosis of the abdominal aorta. In such birds, the hormone did not cause the appear-

*This possibility remains to be demonstrated in the chick.

TABLE LVIII

Effect of DCA on Chick Blood Pressures—Average Mean Blood Pressures in mm. Hg.—Stamler, J. *et al.* (404a)

	Group 1 Plain Mash	Group 2 Plain Mash + DCA	Group 3 Cholesterol Mash	Group 4 Cholesterol Mash + DCA
Series 1				
10 weeks	111	126	126	122
15 weeks	123	142	128	130
Series 2				
5 weeks	135*	157	142	156
10 weeks	152*	165	148	164
15 weeks	153*	178	148	172

*A fifth group, fed plain mash without salt, had blood pressures of 134, 133, 129 mm. Hg. at 5, 10, and 15 weeks respectively, indicating that the salt in the diets of groups 1–4 exerted a slight hypertensive effect

TABLE LIX

Effect of DCA on Spontaneous and Cholesterol-Induced Atherosclerosis—Series 1—Stamler, J. *et al.* (404a)

	% with Lesions in Thoracic Aorta	% with Lesions in Whole Aorta	% with Thoracic Lesions Grade 1 or >	% with Lesions in Whole Aorta Grade 1 or >	Average Gross Grading of Lesions in Birds with Lesions	
					Thoracic Aorta	Whole Aorta
Group 1 *Plain Mash*	0	35	0	0	0	0.4
Group 2—Plain Mash + DCA	0	50	0	0	0	0.6
Group 3—2% Chol. 5% Oil Mash	100	100	100	100	2.4	3.7
Group 4—2% Chol. 5% Oil Mash + DCA	100	100	100	100	2.9	4.2

Chol. is Cholesterol

ance of gross lesions of the induced type in the thoracic aorta. All birds in both plain mash-fed groups (Groups 1 and 2—Table LIX) were free of such lesions.

In cholesterolized birds of Series 1, DCA apparently did not significantly influence the incidence or severity of cholesterol-induced atherosclerosis in either the thoracic or abdominal aorta (Groups 3 and 4—Table LIX). Bruger and Lowenstein also obtained negative results in studies on the effects of DCA on rabbit cholesterol-induced atherosclerosis (504). This absence of a DCA effect in our experiment is possibly related to the failure of the hormone either to intensify hypercholesterolemia or to effect a consistent hypertension. However, atherosclerosis was of such maximal severity in the control group of this pair (Group 3—Table LIX), that any influence of DCA may well have been masked. In view of this possibility, the diet in Series 2 was supplemented with ¼% cholesterol, rather than 2%. Further, in this second series, 1% NaCl was added to the mash of all four groups.

With these alterations in experimental regimens, somewhat different results were obtained. As already indicated, clearcut increments in blood pressure were recorded in both DCA groups. In accord with Series 1, no significant [illegible] were noted [illegible]

birds fed ¼% cholesterol—5% oil mash. Further, as in Series 1, the DCA birds fed plain mash had only a slightly higher incidence and severity of spontaneous atherosclerosis of the abdominal aorta, in comparison with their paired controls (Table LX). Again, no birds in these two groups exhibited cholesterol-induced gross lesions of the thoracic aorta. Unlike Series 1, the DCA-treated, cholesterolized (¼%) birds of Series 2 had an incidence and severity of induced lesions significantly greater than in their paired con-

TABLE LX

Effect of DCA and DCA Hypertension on Spontaneous Atherosclerosis in 1% Salt-Fed Chicks
—Stamler, J. *et al.* (404a)

	Duration weeks	Mean Blood Pressure mm Hg.	% with Thoracic Lesions	% with Lesions in Whole Aorta	% with Lesions Grade 1 or >	Mean Gross Grading of Lesions—Birds with Lesions			Mean Gross Grading of Lesions—All Birds
						thor.	abd	sum	
1% NaCl Mash	10	152	0	0	0	0	0	0	0
1% NaCl Mash + DCA	10	165	0	0	0	0	0	0	0
1% NaCl Mash	15	153	0	33	0	0	0.3	0.3	0 1
1% NaCl Mash + DCA	15	178	0	57	14	0	0 5	0 5	0 3

Thor. is thoracic aorta; abd. is abdominal aorta, sum is whole aorta.

TABLE LXI

Effects of DCA and DCA Hypertension on Cholesterol-Induced Atherosclerosis in Chicks Fed 1% Salt—¼% Cholesterol Mash for 15 Weeks—Stamler, J. *et al.* (404a)

	Mean Blood Pressure mm. Hg.	% with Thoracic Aorta Lesions	% with Lesions in Whole Aorta	% with Whole Aorta Lesions Grade 1 or >	Mean Gross Grading of Lesions—Birds with Lesions			Mean Gross Grading of Lesions—All Birds
					thor.	abd.	sum	
1% NaCl—¼% Cholesterol Mash	148	17	67	0	0.1	0.4	0.5	0.3
1% NaCl—¼% Cholesterol Mash + DCA	172	45	91	37	0.3	0.5	0.8	0.7

Symbols as in Table LX.

trols. This was particularly true of lesions in the thoracic aorta (Table LXI).

In a further experiment, Stamler, et al., studied the effects of cortisone (Compound E of Kendall) on cholesterol-induced atherogenesis in chicks (549). Forty-eight one-day-old cockerels were placed on a diet of chick starter mash supplemented with ½% cholesterol + 5% cottonseed oil (½ C-O diet). At one week of age, 24 birds began to receive a single daily intramuscular injection of 1.5 mg cortisone suspended in saline. The dosage of cortisone was increased stepwise to a maximum of 15 mg. daily during the latter weeks of the experiment.

The principal finding in the cortisone-treated cockerels sacrificed at 12 weeks of age was a significantly greater incidence and severity of gross cholesterol-induced aorta and coronary atherosclerosis. This intensified atherogenesis supervened, although hypercholesterolemia, phospholipemia and C/P ratios were essentially identical in the steroid-treated and control groups (Tables LXII–LXVI).

Cortisone also effected: (1) decreased body weights, despite normal levels of feed intake; (2) increased comb indices and testes weights; (3) slight inconstant elevations of blood pressure The two groups had essentially similar thyroid and adrenal weights, fluid intakes, and plasma levels of glucose, Na and K (Tables LXII and LXIII).

The data of this experiment indicate that cortisone definitely potentiates atherogenesis when accompanied by an atherogenic level of cholesterol intake. Further work is necessary to clarify the causes of this more intensified cholesterol-induced atherogenesis in these corticoid-treated cholesterol-fed birds, particularly since it was associated with little increase in degree of cholesterolemia.

Paterson, et al., studied the effects of cortisone on the spontaneous coronary sclerosis of chicks (696, 697). In accord with its known ability to inhibit fibroblastic activity

(582–584, 598), cortisone effected a significant decrease in the number and size of fibrous intimal plaques. Repeated exposures to stressing stimuli had a similar effect. In view of these results, further research would be of value on the influences of cortisone on the chronic fibrocalcific processes occurring in atheromatous arterial plaques (cf. page 13).

Several workers recently reported studies on adrenal corticoids in rabbits. Kellner and Correll found that adrenalectomy was without significant influence on hypercholesterolemia and atherogenesis in [illegible]

Experiments on the exhibition of cortisone to rabbits on normal diets yielded results somewhat different from those

TABLE LXII

Effects of Cortisone on Cockerels*—Stamler, J. *et al.* (549)

	Control— ½ C—O**	Experimental— ½ C—O + Cortisone
Plasma Na m eq /L	154 0	153.2
Plasma K m eq./L.	6 34	6 02
Blood Glucose mg %	181	165
Water Intake† cc/day	166	129
Blood Pressure mm. Hg.	142	149
Comb Index	17	24

*13 weeks of age—12 weeks on cortisone, 1–15 mg q d.
**½ C—O is ½% cholesterol + 5% cottonseed oil in chick starter mash
†6th week of age.

we observed in cholesterol-fed chicks. Thus, Rich, et al., found that marked lipemia, hypercholesterolemia, glycemia and hepatic lipidosis developed in compound E-treated rabbits (693). Similarly, Gofman observed a marked cortisone-induced rise in plasma levels of S_f 10–20 and higher classes of lipoproteins in rabbits subsisting on normal, non-cholesterol supplemented diets (567). Adlersberg, et al., also observed lipemia, hypercholesterolemia and hyperphospholipemia during cortisone, as well as ACTH, administration to rabbits. No effects were noted with either hormone in dogs (694). None of the foregoing workers studied the effects of cortisone in cholesterol-fed rabbits, nor did they report on aorta and coronary artery findings in their animals.

In view of the minimal general physiological effects observed with cortisone at high dosage levels in chicks, Stamler recently undertook further studies utilizing the adrenal corticoid compound F of Kendall (690). This steroid is far more active than cortisone in inducing hyperglycemia in depancreatized chicks (689) (cf. page 220). Moreover, re-

TABLE LXIII

Effect of Cortisone on Organ Weights of Cockerels*—Stamler, J. *et al.* (549)

	Control— ½ C—O**	Experimental— ½ C—O + Cortisone
Body (grams)	1069	893
Heart (mg)	516	533
Adrenal (mg)	50	46
Testis (mg)	875	1976
Thyroid (mg)	46	48

*13 weeks of age—12 weeks on cortisone, 1–15 mg q d
**½ C—O is ½% cholesterol + 5% cottonseed oil in chick starter mash.

TABLE LXIV

Effect of Cortisone on Plasma Lipids of Cockerels Fed 1/2 C–O Diet*—Stamler, J. *et al.* (549)

	No of Chicks	Total Cholesterol mg %	Lipid Phosphorus mg %	C/P Ratio
Control— ½ C—O	19	260	7.4	36 6
Experimental— ½ C—O + cortisone	12	287	8.1	35.1

*As in Tables LXII and LXIII.

TABLE LXV

Effect of Cortisone on Aorta Atherogenesis of Cockerels Fed 1/2 C–O Diet*—Stamler, J. *et al.* (549)

	No of Birds	% with Lesions	% with Lesions Grade 1 or >	Mean Gross Grading of Lesions—Birds with Lesions	Mean Gross Grading of Lesions—All Birds
Control—½ C—O	19	26	11	0 6	0.1
Experimental—½ C—O + cortisone	12	75	33	1.0	0.8

*As in preceding 3 tables.

cent work suggests that it may be the hormone produced by the normal human adrenal cortex (698, 699). To date, our studies on compound F have embraced only its effects in the depancreatized cholesterol-fed chick (cf. page 220). Twenty 8 week old depancreatized cockerels were divided into two groups, both fed a mash supplemented with ½% cholesterol and 5% cottonseed oil. One group received a daily injection of compound F (1–2 mg.). Unlike cortisone, *this steroid induced profound alterations in this preparation* (Tables LXVII and LXVIII) (cf. its effects on man, references 698, 699). It induced a chronic, insulin-resistant steroid diabetes with glycosuria in some birds (700). It retarded growth markedly, apparently due to its protein catabolic action (698, 699). It effected a significant sustained fall in plasma Na concentration, without influencing plasma K level, water intake, or blood pressure. It grossly intensified cholesterol-induced hyperlipemia (lactescence) and hypercholesterolemia. Despite these alterations in plasma lipids, aorta atherogenesis was not intensified in the steroid-treated, depancreatized cockerels, and coronary atherosclerosis was partially inhibited (Table LXVIII). Further experiments are currently in progress in our laboratory with compound F.

TABLE LXVI

Effect of Cortisone on Coronary Atherogenesis of Cockerels Fed 1/2 C–O Diet*—Stamler, J. *et al.* (549)

	No of Birds	No. with *Lesions*
Control— ½ C—O	9	1
Experimental— ½ C—O + cortisone	9	6

*As in preceding tables.

Finally, an initial study has been completed in this laboratory on the effects of ACTH in intact cholesterol-fed cockerels (701). No significant effects were noted in this preliminary experiment (Table LXIX). Further projects with ACTH are also in progress.

Obviously, the foregoing group of experiments on adrenal cortical and anterior pituitary hormones and atherogenesis represents only a beginning investigation of this highly important field. In addition to the further studies indicated above, exploration is essential of the complex interrelation-

TABLE LXVII

Effect of Compound F in Depancreatized Cholesterol-Fed Chicks—Stamler, J. (690)

	Pancreatectomy ½ C—O*	Pancreatectomy ½ C—O + Cpd. F
Feed Intake (gms/chick/week)	1235	740
Body Weight (gms)	1824	942
Heart Weight (gms)	9 09	5 40
Testis Weight (gms)	5 67	5 05**
Comb Index	44 8	26 6
Thyroid Weight (mg)	80	44
Adrenal Weight (mg)	110	85
H_2O Intake (cc/chick/day)	298	294
Blood Pressure (mm Hg)	146/119	146/122
Plasma Na (m eq /L)	161 9	148.9
Plasma K (m eq /L)	4 48	4.51

*½ C—O is a mash supplemented with ½% cholesterol plus 5% cottonseed oil

**The chicks treated with compound F exhibited a wide range of testis weights.

TABLE LXVIII

Effect of Compound F in Depancreatized Cholesterol-Fed Cockerels—Stamler, J. (690)

	No. of Chicks	Blood Glucose mg %	Plasma Total Cholesterol mg %	% With Aorta Lesions	% With Aorta Lesions Grade 1 or >	Mean Grading of Aorta Lesions	% With Coronary Lesions
Control—Panc. plus ½ C—O*	6	239	692	100	100	3.5	86
Experimental—Panc. plus ½ C—O + Cpd. F	7	410	1502	100	100	3.1	43

*Panc. is pancreatectomy.
½ C—O is mash supplemented with ½% cholesterol plus 5% cottonseed oil.

TABLE LXIX

Effect of ACTH in Cholesterol-Fed Cockerels—Stamler, J. *et al.* (701)

	No of Birds	Plasma Total Cholesterol mg %	% With Coronary Lesions	% With Aorta Lesions	% With Aorta Lesions 1 or >	Mean Grade of Aorta Lesions
Control— Cholesterol + Oil	8	1037	100	100	100	3.1
Experimental— Cholesterol + Oil + ACTH	10	1014	100	100	90	3.3

ships between the hypothalamic-pituitary-adrenal system and the other endocrines (e.g. the thyroid) (505, 534, 702, 703).

H. Biliary Obstruction

Cholesterol is synthesized in the liver, among other sites. Considerable quantities of the sterol are excreted into the gut via the bile (54). Chronic biliary obstruction leads to marked hypercholesterolemia and hyperlipemia in man, and atherosclerosis eventually supervenes.

To our knowledge, no thorough-going studies have been carried out on the influence of hepatic factors on atherogenesis in experimental animals. Chronic biliary obstruction and atherogenesis have not yet been explored in this or other laboratories. Initial reports of experiments in this direction, utilizing rats, have recently been published by Friedman, *et al.* (506–508). Preliminary studies by Stamler, *et al.*, in our laboratory indicate that ligation of the main extrahepatic bile ducts leads to a moderate hypercholesterolemia in chicks (266). Chronic experiments are in order on the various types of atherogenesis in this preparation.

I. Renal Factors

Renal hyperlipemia has been produced experimentally in laboratory animals. Apparently the kidneys play a significant role in the regulation of plasma lipid levels (54, 183, 466–471). A recent report indicates that rabbits and dogs with renal damage rapidly develop cholesterol-induced atherosclerosis (509, 510). Several years ago Dauber, *et al.*, found that chronic renal damage induced in chicks by repeated administration of small doses of uranium salts had little or no effect on cholesterolemia and atherogenesis in plain mash-fed chicks (266). Such experiments in cholesterol-fed chicks remain to be accomplished. As far as we have been able to ascertain, no other studies have been carried out on

spontaneous or cholesterol-induced atherogenesis in animals with renal damage and hyperlipemia. In view of the apparent significance of renal factors in human hypertension and atherosclerosis, further work along this line is strongly indicated.

J. Age

In the Introduction, we briefly reviewed the clinical evidence demonstrating that atherosclerosis is not a disease of aging. In another section* we also summarized the data obtained in our laboratory indicating that both spontaneous and cholesterol-induced atherosclerosis can occur in very young chicks (309) This study of Rodbard, *et al*, yielded another significant finding concerning the apparent influence of age on cholesterolemia and atherogenesis. These workers found that cockerels placed on a 2% cholesterol—5% oil mash at 1 day of age exhibited a plasma cholesterol concentration of 200–500 mg.% during the first seven weeks of life At the eighth week, the cholesterolemia increased spontaneously to a level of 800–900 mg.%, although no change was instituted in the experimental regimen (Figs. 33 and 34). This new high level of cholesterolemia tended to maintain itself during the next 12 weeks. At about the 20th week of age, with the same diet continuing, plasma cholesterol level fell to about 300–500 mg%, remaining in this lower range until about the 26th week. Preliminary data indicate that a secondary rise in cholesterolemia may occur at this time. With the rise in cholesterolemia at the eighth week, incidence and severity of atherosclerosis also increased. Thereafter atherogenesis tended to be progressively more intensified as the period of cholesterol feeding was prolonged (Fig. 34).

The increment and decrement in cholesterolemia of

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*Page 230.

sterol-fed chicks at approximately the 8th and 26th weeks of life respectively suggest the play of endogenous factors influencing lipid metabolism.

To confirm and analyze the eight week phenomenon in greater detail, an additional experiment was made in three groups of chicks. Group 1 was placed on a 2% cholesterol—5% oil mash at hatching; Group 2 on plain mash until the 6th week of life, and then transferred to the cholesterol-oil mash; Group 3 on plain mash for the first seven weeks of life; thereafter the chicks also received cholesterol-oil mash. The plasma cholesterol findings are summarized in Table LXX and Figure 35. The similar high levels of cholesterolemia exhibited by all groups during the 10th week of life cannot be a function of duration of cholesterol feeding and total quantity of sterol consumed over a long period. These almost identical levels of hypercholesterolemia in three

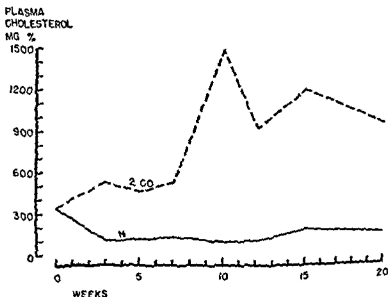


Fig. 33. V[illegible] chicks fed 2% cholesterol [illegible] plain mash [illegible]

groups suggest the operation of age-conditioned endogenous factors which regulate handling of ingested cholesterol. The upward regulation of the plasma cholesterol level during the 8th to the 10th week, occurring regardless of duration of cholesterol feeding, indicates the influence of endogenous alterations in chick metabolism.*

In general, incidence and severity of atherosclerosis in

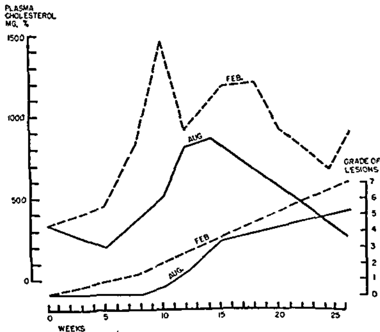


Fig. 34. Variations in cholesterolemia and atherogenesis with age in chicks fed 2% cholesterol mash, two series of chicks, winter (--) and summer (—), for each series the upper curve is cholesterolemia, the lower is grading of lesions—Rodbard, S., *et al.* (309).

*This effect is not detected unless a hypercholesterol[illegible]

this experiment tended to parallel plasma cholesterol concentrations, regardless of duration of cholesterol feeding. Thus while lesions were found earlier in chicks receiving the cholesterol-oil mash since hatching, shortly after the 8th week incidence and severity of cholesterol-induced atherosclerosis became similar in the three groups.

This phenomenon of an age factor in cholesterolemia and atherogenesis is almost certainly not confined to the chick. Thus recent data demonstrate that the plasma cholesterol level in man tends to be higher with advancing age (cf. Table XVI, page 65) (110, 254). Certainly incidence

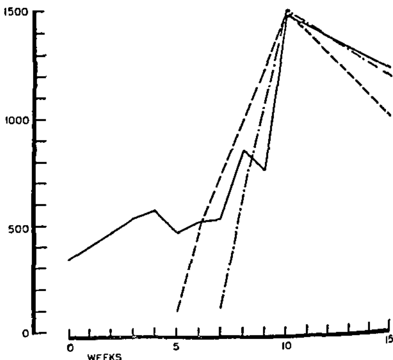


Fig. 35. Cholesterolemic response of chicks placed on 2% cholesterol mash at 0, 5, and 7 weeks of age—Rodbard, S., *et al.* (309).

and severity of human atherosclerosis also increase progressively with advancing age. The experiments of Pollak indicate that a similar general phenomenon is demonstrable in the rabbit (511). He found that under analogous experimental conditions, rabbits one year of age responded to cholesterol feeding with atherosclerosis, whereas 5-, 10- and 25-week-old animals did not. All rabbits, however, regardless of age, reacted with hypercholesterolemia of almost equal degree.

The precise endogenous factors operating to effect such age-conditioned patterns of cholesterolemia and atherogenesis remain to be elucidated. With respect to the cockerel, the 8th week of life is a period of significant changes in endocrine function. These manifest themselves in feather changes and in rapid comb and testicular growth. Thus this age in the chick corresponds to early puberty. At 20 weeks, the chick completes its normal period of growth.

TABLE LXX

Age Factors in Cholesterolemia in Cholesterol-Fed Cockerels —Rodbard, S. *et al.* (309)

	Mean Plasma Total Cholesterol mg %		
	Group 1	Group 2	Group 3
3 weeks of age	524	90	100
5 weeks of age	460	88	76
7 weeks of age	525	476	112
8 weeks of age	840	979	644
10 weeks of age	1467	1471	1481

Hence this age is also a turning point endocrinologically. The possibility presents itself that the alterations in lipid metabolism recorded by Rodbard, *et al.* (309), are related to these changes in hormonal function. Thyroid and adrenal cortical functions may also be involved in these regulations.

In any case, the conclusion is apparent from these experiments that endogenous factors, *conditioned by and varying with age*, influence both the organism's handling of exogenous cholesterol and cholesterol-induced atherogenesis.

K. Sex

In the foregoing discussion we suggested that endocrine factors associated with growth, development and maturation—including sexual maturation—might influence atherogenesis. The possibility may be generally stated, on more than an *a priori* basis, that the gonads affect atherogenesis. In man, the sex differential in susceptibility to coronary atherosclerosis is a key enigma (cf. page 67). Equally intriguing are the recent observations of Gertler, *et al.*, that young males with coronary disease have a low normal 17-ketosteroid excretion (610), and that eunuchs are highly resistant to coronary atherogenesis (656).

In a foregoing section (cf. page 203), recent work in this department was reviewed on the prophylactic and therapeutic inhibition of coronary atherogenesis by estrogens (546, 683). Other experimental work on the role of sex hormones in atherogenesis has been limited. In the chick, Dauber noted that hens frequently exhibit diffuse lipid infiltration of the thoracic aorta. Roosters rarely show this change (300). Later studies revealed that this phenomenon in hens is almost certainly secondary to estrogen-induced hyperlipemia occurring during the egg-laying period of life (292, 293). Dauber also studied the comparative incidence of gross spontaneous atherosclerosis in hens and roosters.

She found it to be essentially identical in the two sexes, namely, 40% for hens and 45% for roosters (300). However, the hens in this series were probably older than the roosters. Further, Dauber classified macroscopic lipid infiltration of the thoracic aorta as an atherosclerotic lesion. These considerations may account in part for the differences between her findings in commercial domestic chickens and those of Fox in wild ground fowl (301, 302). The latter author reported a 9:1 preponderance of spontaneous atherosclerosis in male birds. This latter pattern is in accord with experience in human beings.

In recent preliminary experiments, Rodbard, *et al*, were unable to demonstrate any effect of caponization on hypercholesterolemia and atherogenesis in cholesterol-fed cockerels. Similarly administration of anterior pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) to baby chicks was without clearcut influence (713).

To our knowledge, no further work has been completed on sex factors and atherogenesis in the chick. Further investigation of this problem is currently in progress in our laboratory. A few reports of experiments in the rabbit are available. Several workers found that castration intensified cholesterol-induced atherogenesis in this species (512, 513). In our laboratory, Friedberg, *et al*, found that castrated male and female rabbits exhibited a similar incidence and severity of cholesterol-induced atherosclerosis (266). Turner, *et al.*, reported that cholesterolized male rabbits were less susceptible to atherosclerosis than females (416). Ludden, *et al.*, observed that both androgens and estrogens protected intact female rabbits against hypercholesterolemia and atherosclerosis (514). In the gonadectomized female rabbit, however, hormone administration was ineffective in preventing atherosclerosis (515). It is equally without influence on intact male rabbits. In practically all of these

studies, pathologic examination was confined to the aorta; the effects of the hormones on coronary atherogenesis were not surveyed (cf. pages 203, *et seq.*).

These observations are fragmentary and incomplete. Their interpretation is nigh on to impossible at this time. Obviously this problem of the gonads and atherogenesis merits intense investigation, particularly since data on man demonstrate a definite sex difference in susceptibility to atherosclerosis. In approaching this problem experimentally, consideration must be given to the fact that gonadal function is closely integrated with that of the other endocrines. As in all endocrinological research, primary and secondary effects of experimental interference with specific glands must be distinguished.

L. Species Differences

Among the problems that emerge in the course of studying atherosclerosis experimentally, perhaps the most intriguing is the one of species differences in susceptibility to lesions. Man, the chick and the duck develop atherosclerosis "spontaneously." The dog and the rabbit rarely do.* Experimentally it is easy to produce gross atherosclerosis in rabbits or chicks. It is more difficult in guinea pigs, hamsters and ducks (266, 284, 516, 517). It is still more difficult in dogs (56, 318). To date it has proved impossible consistently to produce gross atherosclerosis in rats. This species resistance of the rat has withstood a diversity of experimental procedures: (1) cholesterol feeding (4); (2) thyroidectomy (266); (3) feeding cholesterol + thiouracil (519); (4) pancreatectomy (266); (5) alloxan diabetes + cholesterol feeding (266); (6) alloxan diabetes + feeding cholesterol and thiouracil (266); (7) unilateral nephrectomy + DCA administration + a high salt, high cholesterol

*The former is an omnivore, the latter a herbivore, subsisting normally on a diet free of cholesterol

diet (266); (8) cholesterol feeding + hyper- and hypovitaminosis E (354).

Why these species differences in atherogenesis? Schlichter, et al., suggested that they may depend on species variability in the adequacy of the vasa vasorum of the large arteries. In a recent study in our laboratory he obtained data indicating that the vascular supply of the aortic wall is richest in the dog, intermediate in man and the rabbit, and poorest in the chick (481, 482). This is the approximate order of "resistance" to the occurrence of atherosclerosis.

Species differences in atherogenesis may also depend on species variability in lipid metabolism (121, 520–523, 555, 566). Studies by Horlick, et al., in this department utilizing the cholesterol tolerance technique (524) suggest that such variations exist (266). Thus the disappearance time of intravenously injected cholesterol is not the same in three species investigated to date. For proportional amounts of cholesterol, the disappearance time in the rabbit is about 72 hours, in the chick 24 hours, in the rat 12 hours. Masson, et al., obtained similar data (525).

In accord with these studies, recent work indicates the existence of species differences in cholesterol-bearing lipoprotein molecules associated with atherogenesis (526). Moreover, investigations utilizing tracer techniques demonstrate species differences between dog and rabbit in cholesterol synthesis, turnover and degradation rates (150, 151). Furthermore, there are marked species differences in the cholesterolemic response to orally ingested cholesterol. Feeding cholesterol to the chick or rabbit results in marked hypercholesterolemia. Proportional amounts have much less effect on the duck (266), and practically no effect on the dog or rat. No data are available from comparable long term cholesterol feeding studies in man (cf. page 32). In the dog, combined thiouracil and cholesterol feeding is necessary to produce marked hypercholesterolemia (56,

318). So far, the rat has proved remarkably resistant to all these regimens. Only moderate hypercholesterolemia has been produced in this species (4, 519), and no gross atherosclerosis. It will be noted that ease of hypercholesterolemia production and ease of atherosclerosis production parallel each other among the various species. Finally, it is noteworthy that only in the rabbit and chick, and perhaps in man, can atherosclerosis be produced with but slight hyper-

TABLE LXXI

Plasma and Red Cell Total Cholesterol Levels in Different Species —Gould, R. G. (555)

	Plasma Cholesterol mg %	Red Cell Cholesterol Red Cell mg %
Rat	52	—
Guinea pig	32	100
Rabbit	50	120
Cat	93	—
Chicken	100	135
Dog	150	140
Sheep	70	145
Hog	120	120
Cow	110	110
Horse	83	122
Human	200	140

cholesterolemia, by feeding small amounts of cholesterol continuously for many weeks (268, 336).

Some investigators tended for a time to minimize the significance of cholesterol-induced atherosclerosis in the rabbit, because this herbivore, having a peculiar lipid metabolism, was for a considerable period the only species susceptible to this experimental lesion. Successful production of atherosclerosis in the chick and the dog by cholesterol feeding compels a reconsideration of this criticism. Undoubtedly further research will clarify the reasons for the foregoing species differences in lipid metabolism and atherogenesis. Concomitantly success will be realized in the experimental production of lesions in resistant species like the rat Precisely through such investigation of the endogenous factors (endocrinological, enzymatic, etc.) responsible for these species differences, will leads be obtained fruitful for the ultimate solution of the whole atherosclerosis problem.

M. Heparin

Alimentary lipemia in dogs was found by Hahn to disappear rapidly following the intravenous injection of heparin (543). This phenomenon is apparently related to the fact that heparin disrupts globulin-lipid bonds, with release of the combined lipids and formation of a heparin-protein complex (544, 672–674).

Lyon, et al, recently studied this effect of heparin by the ultracentrifuge technique (539). They found that in both rabbits and man parenteral heparin shifts the lipoproteins of high S_f classes into those of successively lower classes. "Thus reductions in the S_f 20–100 and S_f 10–20 lipoproteins can be maintained for periods of several hours to days in the human and in the rabbit, depending upon the mode of injection of heparin. The alterations in lipoprotein levels produced by heparin in the rabbit are favorable, in that the

rabbits receiving intermittent heparin injections during cholesterol feeding experiments show definite suppression of development of atherosclerosis" (539). These effects of heparin are well exemplified by Table LXXII from a more recent paper by Graham, *et al.* (675). Block, *et al.*, reported the additional observation that after intravenous heparin, alimentary lipemia clears less rapidly in atherosclerotic as compared with normal males (Table LXXIII) (614).

It should be noted that these heparin-induced alterations in concentration of various plasma lipoproteins occur without any significant concomitant change in plasma total cholesterol level or blood coagulability. Nonetheless, in the cholesterol-fed rabbit, these effects of heparin on plasma lipids are associated with inhibition of atherogenesis (539, 556, 675). Also noteworthy in relation to these observations are the findings of Dedichen and Closs (570, 676). They gave

TABLE LXXII

Effect of Heparin Repository on Serum Low-Density Lipoproteins of Man—Graham, D. M. *et al.* (675)

Time After Heparin	S_f 12–20 mg %	S_f 20–100 mg %
Pre-heparin	197	357
70 min.	147	18
3 hours	102	14
4 hours	21	0
6 hours	83	18
24 hours	81	160
72 hours	107	164

TABLE LXXIII

Heparin-Induced Clearing of Alimentary Lipemia—Block, W. J. *et al.* (614)

Group	Number of Cases	Percentage Clearing			Number Clearing Less than 40%
		Highest	Lowest	Average	
Normal Women	20	100	62	84	0
Normal Men	23	100	28	74	2
Atherosclerotic Male Patients	27	100	0	38	16

stilbesterol-implanted chicks dicumarol, and found a correlation between level of cholesterolemia and coagulability of the blood. Thus dicumarol, unlike heparin, apparently depresses plasma total cholesterol levels. In contrast, ultracentrifuge studies revealed no influence of dicumarol on plasma S_f patterns (567). These actions of dicumarol are also being further explored (676).

Recently Graham, et al., began to treat angina pectoris cases with intermittent heparin injections, with presumably encouraging results (556, 675). Prolonged study is essential to determine the precise therapeutic significance of heparin in clinical atherosclerosis.

WHAT EXPERIMENTAL EVIDENCE IS AVAILABLE CONCERNING THE INFLUENCE OF PLASMA $\frac{\text{TOTAL CHOLESTEROL}}{\text{LIPID PHOSPHORUS}}$ (C/P) RATIOS ON ATHEROGENESIS?

We have already reviewed the clinical evidence suggesting that an elevated plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio is an important factor in human atherogenesis (page 89). The data were collected primarily in patients with coronary atherosclerosis (107–111) and xanthomatous biliary cirrhosis (203–205).

Recently a number of laboratory studies have focussed upon this possibility. Ahrens and Kunkel showed by *in vitro* experiments that clear sera with either normal or high lipid content may be rendered lipemic by enzymatic hydrolysis of serum "lecithin" with *Cl. welchii* lecithinase They pointed out, "The concentration of serum phospholipids available for complex formation with serum proteins appears to be an important factor in determining particle size

of serum lipids and hence of their occurrence in serum as masked or as visible particles" (396). As already noted, their deductions implicating elevated plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratios in atherogenesis are based on clinical correlations between plasma lipid patterns and occurrence of atherosclerosis While stressing the role of serum phospholipids in preventing lipemia (macrochylomicronemia), they avoid or only hint at any hypothesis concerning the significance of plasma lipid particle size for atherogenesis. Thus it is not made clear whether they believe phospholipid protects against atherosclerosis by stabilizing cholesterol in the plasma, or by preventing macrochylomicronemia. Or are these one and the same process?

Cholesterol-induced atherosclerosis in the rabbit (86), the chick (308) and the dog (397) is almost uniformly associated with a disturbed lipid pattern characterized by hypercholesterolemia and elevated plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio. In attempting to assay which of these two changes are decisive for atherogenesis, experimental answers must be found to at least two questions· Does atherogenesis occur without an elevated C/P ratio? Is cholesterol-induced atherogenesis prevented by restoring the C/P ratio to normal in the presence of hypercholesterolemia?

Kellner, et *al.*, attempted to answer these questions by *in vivo* experiments. They studied the effects of intravenous detergents (Tween 80 and Triton A–20) on plasma lipid levels and atherogenesis in rabbits (398–400). These investigators found that intravenous administration of these surface active agents induces a sustained hypercholesterolemia and hyperphospholipemia in rabbits fed a normal diet. Elevations of plasma phospholipid equalled or exceeded increases in cholesterol Apparently, therefore, the plasma

$\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio was not increased. Nonetheless, the plasmas were definitely lipemic (cf. Ahrens and Kunkel, pages 54, 89 and 264). They succeeded in maintaining this hyperlipemia for 8–14 weeks by repeated detergent injections. Atherogenesis was compared in these rabbits and in animals fed cholesterol. The latter rabbits had the typical plasma lipid pattern of cholesterol-induced hyperlipemia, with marked hypercholesterolemia and elevated plasma C/P ratios. It was found that the animals with detergent-induced hyperlipemia had higher plasma cholesterol levels, but significantly less atherosclerosis than the cholesterol-fed rabbits.* In a third group of animals, detergents and a cholesterol diet were both used. This resulted in a proportional elevation of phospholipid concomitantly with cholesterol, establishing approximately normal plasma C/P ratios. In these animals, incidence and severity of atherosclerosis were decreased. The authors suggest, ". . . Increased blood phospholipids may modify or prevent the development of atherosclerosis" (399).† Payne and Duff have recently confirmed these observations (401, 677).

In our opinion the findings in this last group of rabbits are highly suggestive—provided such factors as decreased food intake, weight loss and other possible non-specific effects of the detergents were not associated elements in the decreased incidence and severity of cholesterol-induced atherosclerosis in the detergent-treated rabbits In this regard, Payne and Duff noted that inhibition of atherogenesis in cholesterol-fed, detergent-treated rabbits cannot definitively be attributed merely to plasma phospholipid elevation.

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*This difference in atherogenesis may be a function not of the C/P ratio, but of the varying total amounts of cholesterol handled by the organism in the cholesterol fed vs. the detergent treated group

†They found intravenous detergents to be without value in the therapy of established cholesterol induced atherosclerosis in rabbits.

Since the injected detergent remains in the blood stream for a long time, it is possible that its inhibitory effect on atherogenesis is independent of the plasma lipid changes (677).

In another recent study, Duff and Payne present data implicating increased plasma C/P ratios as key factors in atherogenesis (287). They analyzed plasma lipid values in alloxan diabetic and control rabbits fed cholesterol. They sought by these biochemical studies to account for the unexpected finding of less severe atherosclerosis in the diabetic animals (402). They arrived at the following general conclusion: ". . . In those diabetic rabbits in which there was a marked elevation of neutral fat and lipid phosphorus* in proportion to the increase in total cholesterol, the arteries were protected from the development of atherosclerosis . . . [even though] the level of serum cholesterol in the protected animals was just as high as, or higher than that in the rabbits that were not protected from the development of atherosclerosis" (287).

As already indicated (cf. page 211), the recent ultracentrifuge studies of Pierce on plasmas of cholesterol-fed, alloxanized rabbits indicate that inhibition of atherogenesis is probably related primarily to alterations in plasma lipoprotein patterns (S_f levels), rather than to changes in C/P ratios (556, 678).

Several studies in our laboratory bear upon this problem of C/P ratios and atherogenesis To reiterate, our results demonstrate that cholesterol-induced atherosclerosis is usually associated with both hypercholesterolemia and elevated C/P ratios Investigators interested in the relative importance of each for atherogenesis have, however, completely neglected the findings in chick stilbesterol-induced

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*Duff and Payne suggest that neutral fat and lipid phosphorus are more frequently elevated in alloxanized rabbits fed cholesterol as a result of a metabolic mobilization of body fat into the blood stream, in connection with diabetic semistarvation (L.N.K. and J.S.).

$\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ *ratio was not increased. Nonetheless,* the plasmas were definitely lipemic (cf. Ahrens and Kunkel, pages 54, 89 and 264). They succeeded in maintaining this hyperlipemia for 8–14 weeks by repeated detergent injections. Atherogenesis was compared in these rabbits and in animals fed cholesterol. The latter rabbits had the typical plasma lipid pattern of cholesterol-induced hyperlipemia, with marked hypercholesterolemia and elevated plasma C/P ratios. It was found that the animals with detergent-induced *hyperlipemia had higher plasma cholesterol levels, but significantly* less atherosclerosis than the cholesterol-fed rabbits.* In a third group of animals, detergents and a *cholesterol diet were both used. This resulted in a proportional* elevation of phospholipid concomitantly with cholesterol, establishing approximately normal plasma C/P ratios. In these animals, incidence and severity of atherosclerosis were decreased. The authors suggest, ". . . Increased blood phospholipids may modify or prevent the development of atherosclerosis" (399).† Payne and Duff have recently confirmed these observations (401, 677).

In our opinion the findings in this last group of rabbits are highly suggestive—provided such factors as decreased food intake, weight loss and other possible non-specific effects of the detergents were not associated elements in the decreased incidence and severity of cholesterol-induced *atherosclerosis in the detergent-treated rabbits.* In this regard, Payne and Duff noted that inhibition of atherogenesis in cholesterol-fed, detergent-treated rabbits cannot definitively be attributed merely to plasma phospholipid elevation.

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* [illegible] atherogenesis may be a function not of the C/P ratio, [illegible] the organism in [illegible]

†They found intravenous detergents to be without value in the therapy of established cholesterol induced atherosclerosis in rabbits.

atherosclerosis (292, 293, 403). Estrogens produce a typical alteration in the plasma lipid pattern of cockerels which differs significantly from the hyperlipemia of cholesterolized birds (Table LXXIV). With stilbesterol implantation, hyperphospholipemia (not hypercholesterolemia) is the most prominent feature. Hypercholesterolemia is less marked. The plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio falls. The plasma $\frac{\text{free cholesterol}}{\text{total cholesterol}} \left(\frac{FC}{TC}\right)$ rises.* With these changes, the plasma is lipemic. Atherogenesis in the thoracic aorta proceeds. Under these experimental conditions, a fall in the C/P ratio prevents neither plasma lactescence nor aorta atherosclerosis. Apparently an elevated C/P ratio is not essential for aorta atherogenesis in the chick. Is it possible that atherogenesis *in these stilbesterol-implanted cockerels* is related to the induced depression below the normal range of the plasma C/P ratio?

Another experiment by Stamler and Katz on C/P ratios and atherogenesis attempted to correlate plasma biochemical data and postmortem pathological findings in the aorta of chicks fed a ¼% cholesterol mash for 35 weeks beginning at five weeks of age (336).† The findings are summarized in Tables LXXV and LXXVI. Analysis of these data reveals that cholesterol-induced atherosclerosis in the thoracic aorta—the primary site of this experimental lesion—occurred in 100% of chicks with mean plasma total cholesterol above 175 mg.%. In contrast, birds with plasma cholesterol concentration below 175 mg.% were uniformly

*In our experience, estrogens are the only agents which alter the $\frac{FC}{TC}$ ratio. In cholesterol fed chicks with marked hypercholesterolemia, no significant changes in this ratio are observed Is atherogenesis related to this plasma lipid ratio?

†Coronary lesions were not analyzed in this experiment.

TABLE LXXIV

Plasma Lipid Patterns in Control, Cholesterol-Fed, and Stilbesterol-Implanted Cockerels—Stamler, J. et al. (308, 403)

	Total Cholesterol mg %	Lipid Phosphorus mg %	Plasma C/P Ratio	Plasma FC/TC Ratio**
Controls	101	7 8	13.3	.38
Cholesterol Fed*	332	9 0	36.9	.36
Stilbesterol Implanted+	684	112.0	6.1	.69

* Mash supplemented with 2% cholesterol + 5% oil, fed for 1 week prior to bleeding and analysis
\+ Subcutaneous implantation of a 25 mg. pellet of diethylstilbesterol 1 week prior to bleeding and analysis
**Plasma FC/TC Ratio = Ratio of free cholesterol (FC) to total cholesterol (TC).

this experiment demonstrates a lack of correlation between plasma C/P ratios and atherosclerosis, in association with a positive correlation between plasma cholesterol levels and lesions. This evidence supports the concept that plasma cholesterol levels *per se* are more significant for cholesterol-induced aorta atherosclerosis than C/P ratios.

Recently Stamler, *et al.*, repeated this experiment, with a single variation (404). Chicks were placed on ¼% cholesterol mash at one day of age, instead of five weeks. With this procedure, neither hypercholesterolemia nor C/P ratio elevation was observed during the first 10 weeks. Apparently this level of cholesterol ingestion represents the borderline tolerance dosage for the chick, with respect to development of hyperlipemia. Preliminary data indicate a significant incidence of gross cholesterol-induced atherosclerosis in the thoracic aorta of these birds. Here aorta atherogenesis is correlated with neither hypercholesterolemia nor C/P ratio rise. The apparently decisive factor is rather the increased load of exogenous cholesterol which the birds must assimilate, turnover, metabolize and excrete. Significant for our immediate discussion, however, is the demonstration once again that

TABLE LXXVI

Biochemical-Pathological Correlations in Chicks Fed 1/4% Cholesterol Mash—Stamler, J. and Katz, L. N. (336)

	Mean Plasma Total Cholesterol mg %	Mean Plasma C/P Ratio
Chicks with Thoracic Lesions	206	20.4
Chicks without Thoracic Lesions	153	20 6

free of thoracic aorta atherosclerosis. The mean plasma total cholesterol of five chicks with thoracic lesions was 206 mg.% (range: 179–265); the mean plasma total cholesterol of four birds free of thoracic lesions was significantly lower —153 mg.% (range: 129–170).

In contrast, no such close relationship could be demonstrated between plasma C/P ratios and thoracic aorta atherogenesis (Table LXXVI). The birds with and without thoracic lesions had practically identical mean group values for this ratio, equal to 20.4 and 20.6 respectively (ranges: 16.5–24.8 and 14.5–27.1 respectively). Thus, in summary,

TABLE LXXV

Plasma Cholesterol Levels (mg %) and Incidence of Lesions in Individual Chicks Fed ¼ % Cholesterol—Stamler, J. and Katz, L. N. (336)

	% with Lesions	% with Lesions 1 or >	Average Grading
Thoracic Aorta			
Mean Total Cholesterol > 175	100	60	1.1
Mean Total Cholesterol < 175	0	0	0
Abdominal Aorta			
Mean Total Cholesterol > 175	60	60	1 7
Mean Total Cholesterol < 175	50	25	1.3
Whole Aorta			
Mean Total Cholesterol > 175	100	80	2.2
Mean Total Cholesterol < 175	50	25	1.3

TABLE LXXVII

Mean Plasma Lipid Findings in Cholesterol-Fed (Group 1) and Cholesterol-Fed, Estrogen-Injected (Group 2) Cockerels—Stamler, J. *et al* (405)*

	Total Cholesterol mg %	Lipid Phosphorus mg %	C/P Ratio
Group 1 Cholesterol Only	732	15	48
Group 2 Cholesterol + Estrogen	804	39	21

*Cf. Table L.

TABLE LXXVIII

Mean Grading for Gross Aorta Atherosclerosis in Cholesterol-Fed (Group 1) and Cholesterol-Fed, Estrogen-Injected (Group 2) Cockerels—Stamler, J. et al. (405)*

	No. of Chicks	% with Lesions	% with Lesions Grade 1 or >	Mean Gross Grading of Lesions
Group 1 Cholesterol Only	8	100	100	2.6
Group 2 Cholesterol + Estrogen	8	100	100	3.5

*Cf. Table L.

aorta atherogenesis may proceed with normal plasma C/P ratios.

Finally, the results of our experiments on the effects of estrogens in cholesterol-fed chicks (cf. page 203) also bear upon this problem of C/P ratios and atherogenesis (405, 546). As already indicated, chicks fed cholesterol develop a predominant hypercholesterolemia (C/P ratios elevated), whereas chicks given estrogens exhibit a predominant hyperphospholipemia (C/P ratios reduced) (cf. Table LXXIV). Combination of cholesterol feeding and estrogen administration therefore presents the possibility of inducing a marked hyperlipemia with C/P ratios approaching normal. This anticipated result was obtained when the experiment was undertaken (Table LXXVII).* The establishment of normal or near normal C/P ratios exerted no inhibitory effect on aorta atherogenesis in these birds (Table LXXVIII); coronary atherogenesis was, however, suppressed. Data on individual birds suggest a close relationship between estrogen-induced reduction of C/P ratios toward normal and inhibition of coronary atherogenesis (cf. Figures 29–31 and Table L, pages 205–208) (546) However, it remains a moot question whether this is a cause-and-effect or merely a correlative (conjunctive) association. It may be that the degree of lowering of C/P ratios toward normal values is merely an index of estrogen effect, and that the birds with greater estrogen effect are completely free of coronary lesions independent of C/P ratio changes *per se*

In conclusion, conflicting data are available bearing upon the concept that elevation of the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$

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* [illegible] (cf. page 557).

ing the ensuing weeks, definite regression and healing of cholesterol-induced lesions were observed (Table LXXIX and Fig. 37). Grossly, the lesions in these birds tended to be less yellow in comparison with birds sacrificed after 10–24 weeks of cholesterol feeding. *They were also less extensive and severe* (Table LXXIX). Both the thoracic and the abdominal aorta appeared dull, white and thickened in a number of instances, but showed little or no involvement with atheroma. Microscopically, during weeks 10–15 the lesions were not markedly different from cholesterolized controls in these two sets of birds restored to diets devoid of a cholesterol supplement. During weeks 15–24, the severity of intimal lesions was definitely less. Previously existing, moderately severe atheromas had apparently "healed" and disappeared. Thus some of the chicks sacrificed at the end of

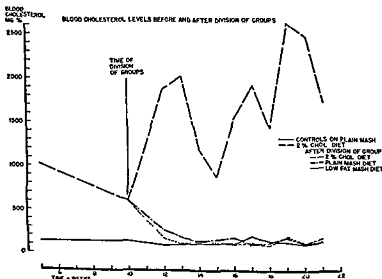


Fig. 36. Effect of prolonged cholesterol feeding vs cessation of cholesterol feeding on cholesterolemia in chicks—Horlick, L. and Katz, L. N. (311).

ratio is a key factor in atherogenesis. Its validity is open to question by the negative results of a number of studies. However, other suggestive positive results, derived from several investigations (including our study of coronary artery *atherogenesis in cholesterol-stilbesterol chicks*), indicate the need for further active exploration of this problem.

ARE ATHEROSCLEROTIC LESIONS REVERSIBLE?

Among the dogmas persisting in the atherosclerosis literature, none is more stubborn than the thesis of the absolute non-reversibility of atherosclerosis. By now considerable clinical evidence has accumulated refuting this notion. It is clear that not only juvenile fatty streaks* (4, 38, 186, 242, 268), but also *more developed* atherosclerotic lesions can regress and be resolved (106, 554).

The experimental data are even more clear cut (268, 311). Horlick and Katz recently demonstrated this in the cholesterol-fed chick. A large group of cockerels was maintained for 10 weeks on a mash supplemented with 2% cholesterol + 20% cottonseed oil. One set of these chicks was then sacrificed as controls to ascertain the incidence and severity of lesions A second set continued to receive the cholesterol-enriched diet for the next 14 weeks. Two other sets were maintained for the next 14 weeks on diets of regular mash† and defatted mash‡ respectively. Serial sacrifices were accomplished during this experimental period.

The cholesterolemic response on cessation of cholesterol feeding is indicated in Figure 36. In the chicks transferred to regular and defatted mash, cessation of cholesterol feeding led to rapid fall of plasma lipids to normal levels. Dur-

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[illegible] between this lesion in children and [illegible] raph.

lesterol ingestion and hypercholesterolemia) (311).* Peterson and Hirst recently made similar observations on regression of cholesterol-induced lesions in chicks (662).

In a recent study, Rodbard, et al., obtained preliminary data indicating that cholesterol-induced gross atherosclerotic lesions in the chick aorta may be resolved very rapidly, i.e., in 5–7 days (704). Extended observations are at present being made in this laboratory on rapid regression of plaques and factors influencing this process.

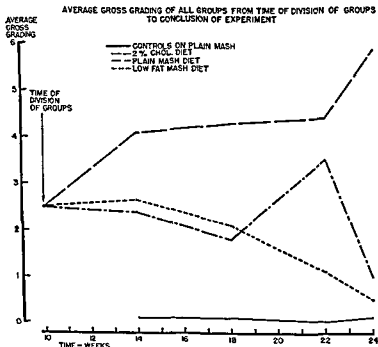


Fig. 37. Effect of prolonged cholesterol feeding vs cessation of cholesterol feeding on gross grading of atherosclerotic lesions in chick aortas—Horlick, L. and Katz, L. N. (311).

*As indicated subsequently (page 280), these sites of healed or metaplased old atherosclerotic lesions *apparently may act as preferential nidi for further* superimposed atherogenesis, when conditions favorable for atherogenesis are re-established.

the experiment showed little gross or microscopic evidence of atherosclerosis. The lesions in these birds had apparently undergone complete remission. In other birds, severe fibrosis, remains of atheromatous abcesses, and heavy calcification suggested that the ability to repair atherosclerotic damage is limited. More severe atherosclerotic lesions apparently undergo only partial regression, with fibrosis, calcification and cartilaginous metaplasia. Under these circumstances the evolution of lesions is apparently influenced in the direction of healing (scar formation) by the absence of continued, freshly superimposed atherogenic stimuli (cho-

TABLE LXXIX

Retrogression of Atherosclerotic Lesions on Cessation of Cholesterol Feeding in the Chick—Horlick, L. and Katz, L. N. (311)*

	Number of Chicks	Average Gross Grading of Lesions
Controls—Cholesterol fed for 10 weeks	18	2 5
Controls—Cholesterol fed for 15–24 weeks	19	4 6
Experimental—Cholesterol fed for 10 weeks, then plain mash for 5–14 weeks	11	2 0
Experimental—Cholesterol fed for 10 weeks, then low fat mash for 5–14 weeks	16	1.2

*Cf. Table XLIII.

retrogressive processes would appear to proceed more rapidly in the chick and dog than in the rabbit. Unfortunately not even a semiquantitative method is currently available to assay rate of regression of human atherosclerotic lesions.

Undoubtedly these findings in chick, dog and rabbit, plus those in man, compel the conclusion that atherogenesis is at least a partially reversible process. Further studies are certainly indicated on the possible influence of various factors (dietary, hormonal) on this healing process. The work of Pick (683) on the reversal of cholesterol-induced coronary atherosclerosis by estrogens (page 206) is an important step in this direction.

DOES ATHEROSCLEROSIS DEVELOP IN EPISODIC STAGES?

The foregoing experiments on prolonged cholesterol feeding and on regression of atherosclerotic lesions also yield data bearing upon the problem of the possible episodic nature of atherogenesis. They suggest that atherogenesis is a discontinuous process even when atherogenic stimuli are continually operating. When these stimuli are intermittent, atherogenesis is certainly intermittent.*

The primary lesion of atherosclerosis (atheroma) is a focal lesion, produced relatively acutely. Once atheroma formation occurs, secondary sclerotic processes supervene. These are slowly developing changes, gradual rather than episodic. They are primarily responses to the tissue irritant effects of cholesterol (8, 530). As such, they are influenced by continuation or withdrawal of atherogenic stimuli Thus

*This conclusion apparently applies to atheroma [illegible]

Other investigators have made similar observations on the effect of suspending cholesterol feeding on the arterial lesions of the rabbit. Stukkeu (19–21), Krylow (527), Wada (528) and Scarff (529) initially reported such observations, but their data were not extensive. Anitschkow (268) was the first to study the problem thoroughly in the rabbit. He found that when rabbits were fed cholesterol for three to four months, and were then returned to a normal diet, a gradual loss of lipids from the plaques resulted. The lesions lost their yellowish color and no longer stained with sudan. They presented a whitish and fibrous appearance Anitschkow stressed that this process was a slow one. The transformation of a large plaque rich in lipids into a plaque composed of fibrous tissue took two to three years in rabbits—in contrast to the much more rapid retrogression (three to four months) in chicks (311). Even after two to three years in rabbits small quantities of lipid could still be discovered histologically after sudan staining. Microscopically Anitschkow observed the gradual disintegration and disappearance of the foam cell masses and their replacement by newly developing collagenous and elastic fibers and fibroblasts. Cholesterol crystals tended to persist long after the other lipids had been removed. Large quantities of calcium were laid down in areas formerly occupied by atheromatous abscesses. Anitschkow also observed that less advanced lesions left minimal residua. After resorption of the lipids only a slight fibrous thickening of the intima remained, with a few cholesterol crystals, scattered lipid cells and globules of neutral fat sometimes fixed *in situ* (268).

Recently, Bevans, *et al.*, further demonstrated that atherosclerotic lesions produced in dogs by the cholesterol-thiouracil regimen regress within 2–4 months after return to normal diet (566, 705). Thus essentially the same phenomenon supervenes in the three species studied, except that these

man differs fundamentally in pathogenesis and etiology from the cholesterol-induced lesion in laboratory animals.

In view of these considerations, Stamler, et al., undertook two long-term experiments to explore this problem further (336, 404a). Their purpose was to determine whether cholesterol-induced atherosclerosis could be produced in the chick with minimal concomitant hypercholesterolemia and organ lipidosis.

In the first of these studies, five-week-old cockerels were placed on a diet of chick starter mash supplemented with ¼% cholesterol + 5% cottonseed oil. They were maintained on this ration for the subsequent 35 weeks. This dietary regimen induced a minimal hypercholesterolemia The over-all mean plasma cholesterol concentration rose from a control value of 99 to 166 mg.% (Table LXXX). Over the 35 weeks of the experiment, periodic plasma lipid analyses yielded mean group values for the experimental birds ranging from 116 to 247 mg.% (controls: 68–142 mg.%). The minimum and maximum values for individual experimental chicks were 78 and 395 mg.% respectively (controls: 45 and 230 mg %). All these changes in plasma

TABLE LXXX

Effect of Feeding ¼ % Cholesterol on Plasma Lipids in the Chick*
—Stamler, J. and Katz, L. N. (336)

	Total Cholesterol mg %	Lipid P mg %	Total Cholesterol / Lipid P
Control	99	7.5	13 1
Cholesterol Fed	166	7 8	21.4

*Chicks fed ¼% cholesterol—5% cottonseed oil mash from 5th through 40th weeks of life.

persistent hypercholesterolemia, by increasing the amount of cholesterol requiring removal from the lesion site, affects not only the production of primary atheromatous lesions, but also their secondary evolution.

Further, atheroma formation occurs focally, i.e., at different sites at different times. Hence animals fed cholesterol for long periods exhibit (at any one time) lesions in all morphologic stages of development. Their aortas show fresh atheromas adjacent to, *as well as superimposed on*, old, fibrosed, calcified lesions (268, 311, 531). Similar observations have been frequently made in human specimens (56). Thus Wilens recently showed that the edges of old atherosclerotic plaques are susceptible to fresh atheroma formation, this being the chief basis for progressive enlargement of lesions (706). These pathologic facts support the concept that atherogenesis is a focal, discontinuous, episodic process, with acute (primary) and chronic (secondary) phases.

CAN EXPERIMENTAL ATHEROSCLEROSIS BE INDUCED WITHOUT GROSS HYPERCHOLESTEROLEMIA AND ORGAN LIPIDOSIS?

In many of our experiments, the dosages of cholesterol utilized resulted in marked hypercholesterolemia and organ lipidosis. In contrast, human atherosclerosis frequently occurs in people with presumably normal or near normal plasma and tissue cholesterol concentrations. Those who question the significance of experimental cholesterol-induced atherosclerosis in rabbits and chicks stress this difference. They maintain that the pathophysiology of cholesterolized, hyperlipemic animals has its counterpart only in grossly hypercholesterolemic man. Since gross hypercholesterolemia is not present in most people with clinical atherosclerosis, these critics suggest that atherosclerosis in

the abdominal aorta, these lesions were significantly greater in extent and severity in the chicks fed the ¼% cholesterol mash.

In the second, more recent experiment (404a), Stamler, *et al.*, placed chicks on this ¼% cholesterol—5% cottonseed oil mash at one day of age. With this modification of the experimental procedure, the cholesterolized birds maintained normal plasma cholesterol concentrations throughout the first 10 weeks of the experiment. Thereafter they had slight hypercholesterolemia (Table LXXXIII). With institution of cholesterol feeding at one day of age, chicks were apparently able to handle the sterol more adequately than birds placed on the experimental diet when five weeks old. Despite normocholesterolemia, chicks sacrificed at 10 weeks of age showed early atheroma formation, particularly in the thoracic aorta. At 15 weeks, more advanced cholesterol-induced lesions were seen (Table LXXXIV).

These experiments demonstrate that significant cholesterol-induced atherogenesis proceeds in chicks fed a dietary level of sterol inducing little or no hypercholesterolemia and organ lipidosis.

This finding in the chick is in accord with previous experimental work on the rabbit. In 1933, Anitschkow reviewed the experiments demonstrating that atherosclerosis can be induced in the rabbit without gross hypercholesterolemia and organ lipidosis (268). Summarizing these results, he stated: "Thus, comparatively acute atherosclerotic changes are produced in rabbits by feeding them with cholesterin for three to four months, whereas the analagous [illegible]

smaller quantities of some food that is not too rich in cholesterin, such as milk, we can produce typical atherosclerotic changes in their arteries, while the lipid deposits in the in-

cholesterol concentration, although slight, were significant. No significant alterations in plasma lipid phosphorus concentration were recorded in these birds fed a ¼% cholesterol diet. Hence the plasma $\frac{\text{total cholesterol}}{\text{lipid phosphorus}}$ ratio was moderately elevated, from a control mean value of 13.1 to 21.4 (Table LXXX). The plasma $\frac{\text{free cholesterol}}{\text{total cholesterol}}$ ratio remained unaltered in the experimental cockerels. Plasma total fatty acids and total lipids were slightly elevated as a result of the experimental diet.

Lipid analyses of carcass, lung, adrenal, gut, kidney, heart, aorta and liver were accomplished by Stamler, et al. Most of the organs showed little or no cholesterosis (Table LXXXI). The liver was the only organ exhibiting gross alterations from control tissue lipid levels. Hepatic cholesterol concentration increased four to five fold; a rise in esterified cholesterol accounted for the greater proportion of this increase. Only moderate alterations in the other liver lipid fractions were noted, and the over-all pattern of hepatic lipids was such that total lipids were at normal levels. Thus, in summary, these chicks fed a ¼% cholesterol diet had a minimal hypercholesterolemia and hepatic cholesterosis.

This experimental regimen had a profound effect on atherogenesis, despite the minimal changes in plasma and tissue lipid concentrations. These are summarized in Table LXXXII. Particularly prominent is the high incidence of moderately severe gross atherosclerosis of the thoracic aorta in the experimental chicks. This is in significant contrast to the complete absence of atherosclerosis of the thoracic aorta in all control birds. The gross and microscopic morphology of these lesions was typical of cholesterol-induced atherosclerosis. In addition, while both experimental and control groups had a similar incidence of gross atherosclerosis of

Free Chol mg %	Esterified Chol mg %	Ratio Free: Total Chol	Ratio T. Chol· Lipid P §	Esterified Chol Fatty Acid‖ mg %	Total Fatty Acid mg %	Neutral Fat Fatty Acid¶ mg %	Total Lipid** mg %
109	7	94	5 7	5	6710	6343	7004
109	16	88	3 6	12	7080	6469	7501
468	9	.98	6 7	7	3760	2510	4849
532	36	.94	5.7	26	1715	310	2963
515	642	.45	8.4	468	5690	2824	8029
468	722	.38	8 0	526	5460	2354	7945
236	10	.92	4 9	7	4750	3860	5431
387	34	92	6 0	25	5460	4220	6481
242	10	96	3 7	7	4910	3755	5726
296	47	.85	3.4	34	2165	362	3379
147	8	.95	1 9	6	12790	11323	13664
147	11	93	1 8	8	9180	7622	10098
168	6	97	5 1	4	—	—	—
156	13	92	4 1	10	—	—	—
277	10	.97	3.4	7	5340	3861	6354
621	769	.45	10.5	522	3613	0	5372
149	31	.83	7 7	23	—	—	—
149	21	88	3 1	15	—	—	—
169	23	.88	—	17	—	—	—
586	196	.75	7 1	143	2660	610	4395
291	29	91	—	—	—	—	—
735	285	72	—	—	—	—	—

** Total Lipid = phospholipid + total cholesterol + esterified chol fatty acid + neutral fat fatty acid.

TABLE LXXXI

Tissue Lipid Concentrations in Control (Group 1) and Experimental (Group 2) Chicks —Stamler, J. and Katz, L. N. (336)

Group	Organ	Weeks on diet	Lipid P mg %	Phospholipid* mg %	Phospholipid Fatty Acid† mg %	Total Chol ‡ mg %
I	Carcass	15	20.8	540	362	116
II	Carcass	15	34.4	895	599	125
I	Lung	15	71.4	1855	1243	477
II	Lung	15	99.6	2059	1379	568
I	Adrenal	15	138	3580	2398	1157
II	Adrenal	15	148	3875	2580	1190
I	Gut	15	50.6	1318	883	246
II	Gut	15	69.8	1815	1215	421
I	Kidney	15	65.8	1712	1148	252
II	Kidney	15	101	2640	1769	343
I	Heart	15	83.8	2180	1461	155
II	Heart	15	88.8	2310	1550	158
I	Aorta	15	33.8	880	589	174
II	Aorta	15	41.0	1070	707	169
I	Liver	15	84.5	2199	1472	287
II	Liver	15	133	3460	3318	1390
I	Aorta	25	23.5	612	410	180
II	Aorta	25	54.0	1408	990	170
I	Liver	25	—	—	—	192
II	Liver	25	110	2860	1906	782
I	Liver	35	—	—	—	320
II	Liver	35	—	—	—	1020

* Lipid P × 26.
† Phospholipid × .67.
‡ Chol. is cholesterol
§ Ratio of plasma total cholesterol to plasma lipid phosphorus c/p ratio.
‖ Esterified Chol × .73
¶ Neutral Fat Fatty Acid = total fatty acid — (phospholipid fatty acid + esterified cholesterol fatty acid).

ternal organs and the hypercholesterinemia do not reach a very high degree. In other words, the total picture of the changes produced in the rabbit under the experimental conditions just described resembles that of human atherosclerosis much more closely than that observed during experiments of a shorter duration, in which the rabbit is literally swamped with cholesterin" (268).

When these findings in rabbit and chick are evaluated in relation to the latest data on man, they assume even greater significance. Today a mass of data exists demonstrating that a "xanthomatous tendency" (minimal hypercholesterolemia) prevails in many people with atherosclerosis. More-

TABLE LXXXIII

Plasma Total Cholesterol of Chicks Fed ¼ % Cholesterol Mash During First 15 Weeks of Life—Stamler, J. et al. (404a)

	Plasma Total Cholesterol	
Age	Group I Control mg %	Group II ¼% Cholesterol mg %
3	—	117
5	87	100
8	78	123
10	89	109
12	83	175
15	113	217
Mean	90	140

TABLE LXXXII

Effect of Feeding ¼ % Cholesterol *Mash* on Atherogenesis in the *Chick**
—Stamler, J. and Katz, L. N. (336)

	% with Lesions	% with Grading 1 or >	Average Grading of Lesions
Thoracic Aorta—Controls	0	0	0
Thoracic Aorta—Cholesterol Fed	42	33	1.1
Abdominal Aorta—Controls	50	30	1.0
Abdominal Aorta—Cholesterol Fed	67	58	1.4
Whole Aorta—Controls	50	30	1.0
Whole Aorta—Cholesterol Fed	83	67	1.8

*Chicks fed experimental diet from 5th to 40th week of life

ternal organs and the hypercholesterinemia do not reach a very high degree. In other words, the total picture of the changes produced in the rabbit under the experimental conditions just described resembles that of human atherosclerosis much more closely than that observed during experiments of a shorter duration, in which the rabbit is literally swamped with cholesterin" (268).

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3	—	117
5	87	100
8	78	123
10	89	109
12	83	175
15	113	217
Mean	90	140

TABLE LXXXIV

Aorta Atherosclerosis in Chicks Fed ¼% Cholesterol Mash During First 15 Weeks of Life —Stamler, J. *et al.* (404a)

	Duration Weeks	% with Thoracic Lesions	*% with* Lesions in Whole *Aorta*	*% with* Lesions Grade 1 or >	Mean Gross Grading of Lesions Birds with Lesions			*Mean Gross* Grading of Lesions—All Birds
					thor.	abd.	sum	
Plain Mash	10	0	0	0	0	0	0	0
¼% Cholesterol Mash	10	25	38	13	0.6	0.3	0.9	0.2
Plain Mash	15	0	33	0	0	0.3	0.3	0.1
¼% Cholesterol Mash	15	17	67	0	0.1	0.4	0.5	0.3

See symbols in Table LX.

over, considerable evidence is available indicating that cholesterol "input-load" (the quantity of ingested cholesterol per unit time that the body must absorb, transport, metabolize, turnover and excrete) influences atherogenesis in man.

This conclusion receives further support from the recent studies of Gofman and his associates (152). As already noted, these workers demonstrated by ultracentrifuge methods that atherosclerosis in humans and rabbits is associated with the presence in plasma of a particular class of cholesterol-bearing lipoprotein molecules. Significant for our present discussion is the fact that in man the serum concentrations of this S_f 10–20 class of lipoproteins are influenced by diet.* Partial dietary restriction of fat and cholesterol gradually lowers their serum level over a course of weeks and months. This change occurs with or without any concomitant alteration in serum total cholesterol concentration. Does exogenous cholesterol ingestion elevate the serum S_f 10–20 lipoprotein concentration in man? Is this *the mechanism whereby diet influences atherogenesis in* man, without grossly altering serum total cholesterol concentration? Does a similar mechanism operate in minimally hypercholesterolemic or normocholesterolemic chicks developing atherosclerosis on a ¼% cholesterol mash? Certainly intensive research along these lines is indicated.

*They are not affected during an acute postprandial alimentary hyperlipemia (152).

V CONCLUSION

A BASIC PROPOSITION HAS BEEN THE FOUNDATION OF THE work of this department on experimental atherosclerosis. The essence of this proposition is that altered cholesterol metabolism plays a key role in human atherogenesis. In the words of Anitschkow, "cholesterin is the 'materia peccans'" of atherogenesis (268).

The corollary of this proposition is that study of experimental cholesterol-induced atherosclerosis—of the exogenous and endogenous factors controlling it—will yield knowledge of fundamental significance for our understanding of human atherosclerosis.

For many years some investigators rejected these concepts because atherosclerosis had been produced experimentally only in rabbits. The consistent production of gross cholesterol-induced atherosclerosis in omnivorous chicks and dogs, among other species, now compels rejection of this criticism.

For many years some investigators maintained that experimental and clinical atherosclerosis were fundamentally different, since hypercholesterolemia was a prerequisite for the former, whereas the latter occurred in many normocholesterolemic persons. The consistent production of gross cholesterol-induced atherosclerosis in chicks and rabbits with minimal hypercholesterolemia or normocholesterolemia now compels rejection of this criticism.

Finally, recent investigations on human atherosclerosis,

as reviewed in this monograph, have consistently yielded detailed evidence reinforcing the basic proposition that atherosclerosis in man is consequent upon an altered cholesterol-lipid-lipoprotein metabolism.

As Leary forcefully stated almost 10 years ago, "The accepted criteria for the establishment of a causal relation between a given agent and a disease are embodied in Koch's laws. . . . In the causation of atherosclerosis the principles of Koch's laws can be fulfilled. Excess cholesterol [or altered cholesterol metabolism]* is always present in the active stages of human atherosclerosis It can be identified in the lesions as definitely as can the bacterial or other parasitic agents producing infections. It can be extracted from the lesions. Human arterial lesions can be reproduced experimentally by its use with more exactness than the lesions of many human infections can be reproduced by the introduction into susceptible animals of their recognized causal agents. It can be identified in and extracted from the experimental lesions" (7).

The record of the last decade—of our experiments and the extensive literature reviewed in this monograph—fully corroborates this thesis.

What conclusion must be drawn from this? Certainly present knowledge does not permit us to state that the atherosclerosis problem is solved. Certainly much basic clinical and laboratory research lies ahead before a solution is reached What present knowledge affords us is a fundamental approach for future research—the approach embodied in what we have termed the "cholesterol concept of atherogenesis." In this direction, we predict, lies the solution of the atherosclerosis problem—and with it the eventual control and elimination of this malevolent disease.

*Our addition (L N K. and J.S).

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SUBJECT INDEX

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This Book

experimental
ATHEROSCLEROSIS

By
LOUIS N. KATZ, M.D.
and
JEREMIAH STAMLER, M.D.

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